Human recombinant antibodies against *Mycobacterium tuberculosis* antigens

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Abbrevations

Å	Ångström
α	anti
A	adenine
A_{450}	absorption at 450 nm (620 nm reference)
ADCC	antibody dependant cellular cytotoxicity
AlaDH	alanine dehydrogenase
amp	ampicillin
APS	ammonium persulfate
bp	base pairs
BSA	bovine serum albumin
C	cytosine
CDC	complement dependant cytotoxicity
CDR	complementarity determining region
CFP-10	culture filtrate protein 10 kDa
cfu	colony forming units
C_H	constant domain of the heavy chain
C_L	constant domain of the light chain
D	diversity gene segment
DNA	deoxyribonucleic acid
dntp	deoxyribonucleoside triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ESAT-6	early secretory antigenic target 6 kDa
Fab	fragment antigen binding
Fc	fragment crystallizable
FCS	fetal calf serum
fo	forward
Fv	fragment variable
G	guanine
g	gravity constant
GC	gas chromatography
H	heavy
HAL	human antibody library
HEK	human embryonic kidney
his	histidine
HJ	joining gene segment of the heavy chain
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
HV	variable gene segment of the heavy chain
IFN-γ	interferon gamma
Ig	immunoglobulin

IGRA	interferon gamma release assay
IMAC	immobilized-metal affinity chromatography
IPTG	isopropyl β-D-1-thiogalactopyranoside
κ	kappa
Kan	kanamycin
kb	kilo bases
kDa	kilo Dalton
λ	lambda
L	light
lac	lactose
LAM	lipoarabinomannan
LFIA	lateral flow immuno assay
LJ	joining gene segment of the light chain
LTBI	latent tuberculosis infection
LV	variable gene segment of the light chain
M*. bovis* BCG	*Mycobacterium bovis* bacilli Calmette-Guérin
mRNA	messenger ribonucleic acid
MS	mass spectroscopy
MTP	micro titre plate
Mtb	*Mycobacterium tuberculosis*
NAAT	nucleic acid amplification technique
Ni-NTA	nickel nitrilo tri acetic acid
OD_{600}	optical density at 600 nm
ori	origin of replication
ORF	open reading frame
OSP	osmotic shock fraction
p.a.	per analysis
PAA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pelB	N-terminal leader peptide
pen	penicillin
pH	the negative decadic logarithm of the activity of the (solvated) hydronium ions
P_{lacZ}	Lac promotor
PMSF	phenylmethanesulfonylfluoride
POC	point of care
PP	polypropylene
PPP	periplasmic enriched fraction
PTB	pulmonary tuberculosis
PVDF	polyvinylidene fluoride
rv	reverse
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
scFv	single-chain variable fragment
scFab	single-chain fragment antigen binding

scFv-Fc	single-chain variable fragment fused to Fc-part
SDS	sodium dodecyl sulfate
SEC	size exclusion chromatography
SEM	scanning electron micrograph
strep	streptomycin
T	thymine
Tet	tetracycline
TAE	tris acetate EDTA
TB	tuberculosis
TBE	tris buffered saline
TEMED	N,N,N',N'-tetramethyl-ethane-1,2-diamine
TMB	3,3′,5,5′-tetramethylbenzidine
tris	tris-(hydroxymethyl)-aminomethane
TST	tuberculin skin test
U	unit
UV	ultra violet
V	variable
V_H	variable domain of the heavy chain
V_L	variable domain of the light chain
w/v	weight per volume
WHO	world health organisation
X-ray	radiography

1 Abstract

1.1 Abstract

Tuberculosis (TB) remains a threat to public health. Diagnostic of TB is often expensive or time-consuming. The use of recombinant antibodies for direct detection of *M. tuberculosis* (Mtb) antigens in human specimens could facilitate a simple point of care TB test. A diverse series of antigens was evaluated for this task, including 16 kDa, ESAT-6, CFP-10, LAM, AlaDH, 85 A, 85 B and 85 D. Twenty-two recombinant antibodies against 16 kDa, CFP-10, 85 A, 85 B and 85 D were generated by panning of human naïve phage display libraries. A recombinant α-LAM antibody was generated by isolation of the variable gene segments of the IgM encoding sequences from a hybridoma clone. These antibodies were purified as scFv from *E. coli* or scFv-Fc (scFab-Fc) from HEK293-6E cells, and characterized in detail. It was found, that the α-LAM antibody was only producible in a multivalent scFab-Fc format. Seven antibodies showed a supposed preferential germline gene combination against the 16 kDa antigen. Sandwich detection of 16 kDa and CFP-10 was not possible. Specific antibodies against 85 A, 85 B and 85 D were isolated, allowing for the first time discrimination of individual components of the 85 complex. Furthermore, sandwich detection of recombinant antigen 85 A or 85 B was possible. However multimeric conformation of the antigen was enhancing the recognition. Lack of multimers reduced binding to a minimum. Although no sandwich detection of Mtb samples was possible, a functional α-85 B immuno blot assay was developed. In summary, a variety of antibodies was generated with potential for further development of a TB diagnostic assay or for use in infection research, drug development or passive immunization.

1.2 Zusammenfassung

Tuberkulose (TB) ist immer noch eine Bedrohung für die öffentliche Gesundheit. Die Diagnose von TB ist häufig teuer oder zeitaufwendig. Der Gebrauch von rekombinanten Antikörpern (AK) zur direkten Detektion von *M. tuberculosis* (Mtb) Antigenen in humanen Proben könnte einen simplen TB Schnelltest ermöglichen. Eine mannigfaltige Serie von Antigenen wurde für diesen Zweck untersucht, einschließlich 16 kDa, ESAT-6, CFP-10, LAM, AlaDH, 85 A, 85 B und 85 D. Zweiundzwanzig rekombinante AK gegen 16 kDa, CFP-10, 85 A, 85 B und 85 D wurden mittels Panning von humanen naïven Phagendisplaybibliotheken generiert. Ein rekombinanter α-LAM AK wurde generiert durch Isolierung der variablen Gensegmente der IgM kodierenden Sequenzen eines Hybridoms. Diese AK wurden als scFv aus *E. coli* oder als scFv-Fc (scFab-Fc) aus HEK293-6E Zellen aufgereinigt und detailliert charakterisiert. Der α-LAM AK war nur als multivalenter scFab-Fc produzierbar. Sieben AK zeigten eine bevorzugte Genkombination gegen das 16 kDa Antigen. Ein Sandwichnachweis von 16 kDa und CFP-10 war nicht möglich. Spezifische AK gegen 85 A, 85 B und 85 D konnten isoliert werden, die erstmals eine Unterscheidung individueller Komponenten des 85 Komplexes ermöglichen. Weiterhin war der Sandwichnachweis von rekombinantem 85 A oder 85 B möglich, wobei eine multimere Antigenkonformation die Erkennung verbesserte. Das Fehlen von Multimeren reduzierte die Bindung auf ein Minimum. Ein Sandwichnachweis von Mtb Proben war nicht möglich, jedoch konnte ein funktionaler α-85 B Immunoblottest entwickelt werden. Insgesamt wurden eine Vielzahl von AK generiert mit Potential für die weitere Entwicklung eines TB Schnelltests, für die Verwendung in der Infektionsforschung, für die Medikamentenentwicklung oder zur passiven Immunisierung.

2 Introduction

2.1 Tuberculosis

Tuberculosis (TB) is an infectious disease, spread through the air, which is caused by various strains of mycobacteria. Besides *Mycobacterium tuberculosis* (*M. tuberculosis*, Mtb), the main cause of TB in humans, the *M. tuberculosis* complex includes minimum four other human-pathogenic mycobacteria: *M. bovis*, *M. africanum*, *M. canettii*, and *M. microti* (van Soolingen *et al.*, 1997; Brosch *et al.*, 2002). *M. bovis* causes TB in cattle and was once a common cause of TB in humans due to consummation of unpasteurized milk (Kumar *et al.*, 2007; Thoen *et al.*, 2006). *M. africanum* is not widespread, but it is a significant cause of TB in parts of Africa (Niemann *et al.*, 2002). *M. canettii* is rare and seems to be limited to the Horn of Africa (Acton, 2011; Pfyffer *et al.*, 1998; van Soolingen *et al.*, 1997). *M. microti*'s natural hosts are small rodents such as voles, shrews and wood mice, nonetheless it can cause TB in immune deficient humans (Panteix *et al.*, 2010).

Predominantly a disease of the lung, so called pulmonary TB (70 % of the cases), the pathogen can infect any organ system (extrapulmonary TB) like the heart, bones, joints, gastrointestinal system, genitourinary system, central nervous system, mastoid processes and eyes (Harisinghani *et al.*, 2000). The combination of symptoms in pulmonary TB may range from systemic responses such as weight loss ("consumption"), fever and night sweats, to local consequences of the infection such as cough and haemoptysis (Brändli, 1998) to radiological abnormalities (Figure 1) such as thoracic lymphadenopathy and lung cavities or densities (O'Garra *et al.*, 2013).

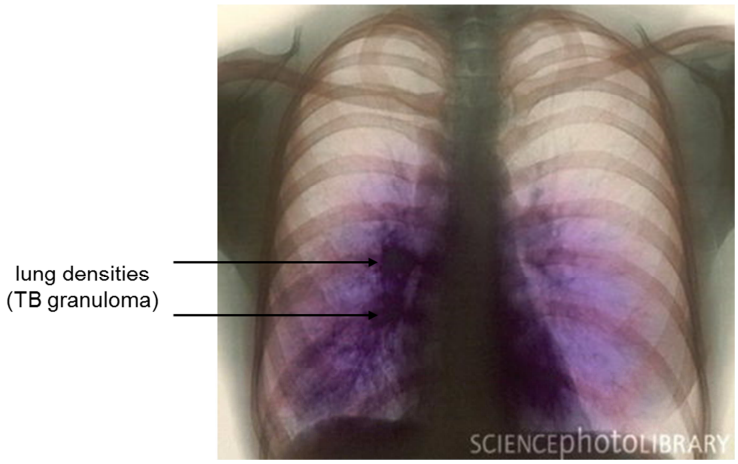


Figure 1: Coloured chest X-ray of a woman with pulmonary tuberculosis.
In retrospect lung densities (consisting of TB granulomas) were highlighted in purple. Figure adapted from science photo library, M270/0179, Simon Fraser.

According to the World Health Organisation (WHO, 2012) approximately 8.7 million people fell ill with TB in 2011 and 1.4 million deaths were reported worldwide. Therefore TB ranks as the second cause of death from an infectious disease worldwide, after the human immunodeficiency virus (HIV). In addition to that one third of the world population is estimated to be infected with Mtb, yet they remain asymptomatic, defined as latent TB infection (LTBI) (Dye *et al.*, 1999). LTBI is specified by the presence of an immune response directed against Mtb antigens and not by the confirmed presence of the pathogenic bacilli. Some LTBI individuals may progress to postprimary active TB after years of subclinical infection, others may maintain a persistent lifelong asymptomatic infection and some are able to effectively clear the pathogen (O'Garra *et al.*, 2013).

2.1.1 M. tuberculosis

M. tuberculosis was first discovered in 1882 as the etiology of TB by Robert Koch (Koch, 1882). This facultative intracellular parasite is a rod shaped, aerob, non-motile, gram positive and acid-fast bacterium of max. 0.5 x 4 µm size which divides only every 15 – 20 hours (Mandell *et al.*, 2009). Mycobacteria possess a complex cell envelope (Figure 2), that consists of a thick asymmetric lipid bilayer located beyond the plasma membrane, peptidoglycan, and an arabinogalactan layer esterified with mycolic acids (mAG) (Umesiri *et al.*, 2010). Proteins and polysaccharides are found in the outermost stratum known as the capsule, and somewhere interspaced lipopolysaccharides such as lipoarabinomannan (LAM) are located (Brennan, 2003). The extremely hydrophobic cell envelope forms a permeability barrier that prevents entry of many hydrophilic solutes, thereby making these bacteria acid-fast and able to survive extremely hostile surroundings (Favrot and Ronning, 2012).

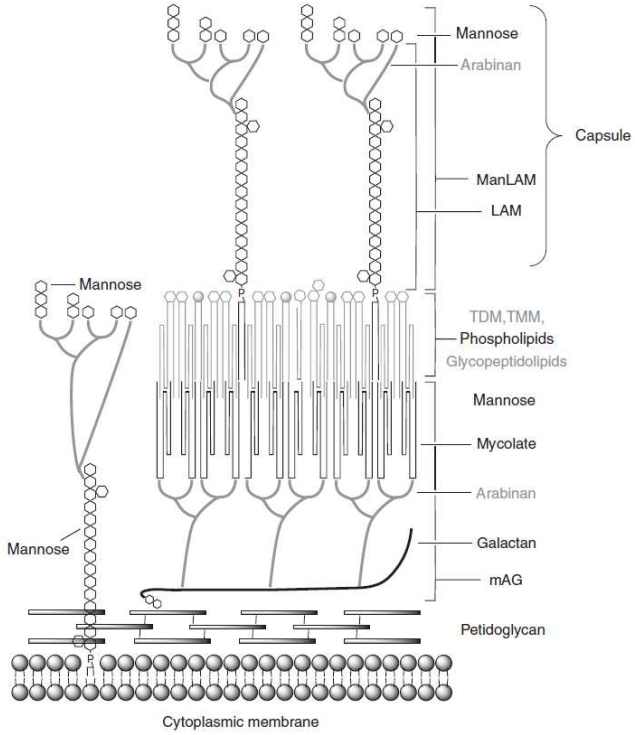


Figure 2: Schematic representation of the general structure of the mycobacterial cell envelope.
LAM: lipoarabinomannan, ManLAM: mannose-capped LAM, TDM: trehalose dimycolate, TMM: trehalose monomycolate, mAG: branched arabinogalactan polymer esterified with mycolic acids. Figure adapted from Umesiri *et al.*, 2010.

2.1.2 Pathogenesis

When a TB infected individual coughs, sneezes or spits, contagious aerosols can be inhaled into the airways and alveoli of a new host (Fennelly *et al.*, 2012). The mycobacteria are then phagocytosed by alveolar macrophages, dendritic cells (Schlesinger, 1996) and neutrophils (Wolf *et al.*, 2007). The following immune response to Mtb is complex and incompletely characterized. Mtb can be eliminated by macrophages via different mechanisms (Pieters, 2008) but it is also known that Mtb can suppress autophagic pathways in macrophages and survive and replicate within the phagosome (Deretic *et al.*, 2009). In dendritic cells the pathogen is carried to the lung-draining lymphnodes, where Mtb–derived antigens are presented to T-lymphocytes and Mtb antigen-specific T cells are generated (Wolf *et al.*, 2008). These cells recirculate and induce formation of granulomas in the lung (Cayabyab *et al.*, 2012). Human TB granulomas (“tubercle”, Figure 3) consist of a central mass of infected macrophages surrounded by a variety of lymphocytes, largely T cells, B cells, fibroblasts and dendritic cells (Ehlers and Schaible, 2013). Granulomas can restrain bacterial cells from spreading and slow down their physiological dynamics, but often the bacilli are not eliminated and latency is promoted (O'Garra *et al.*, 2013).

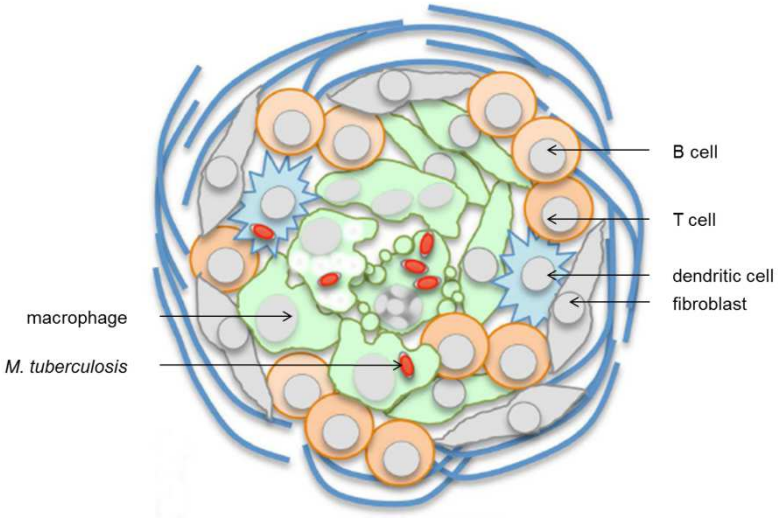


Figure 3: Schematic composition of a protective granuloma.
Human TB granulomas are formed in the lung as a tissue reaction to limit bacillary growth and sequester infection. Granulomas consist of a central mass of infected macrophages surrounded by a variety of lymphocytes, largely T cells, B cells, fibroblasts and dendritic cells. Figure modified after Ehlers and Schaible, 2013.

2.1.3 Vaccination

The only available vaccine against TB remains *M. bovis* bacilli Calmette-Guérin (*M. bovis* BCG), which was generated in 1920 after continual passaging of the parental strain *M. bovis* for 13 years resulting in an attenuated strain with reduced virulence (Calmette 1931). BCG is a living vaccine, applied as a single intradermal inoculation dose, routinely administered to infants in many countries worldwide (Ottenhoff et al. 2012). It provides protection against miliary TB and meningitis TB in infants (Trunz et al. 2006), but fails to protect against pulmonary and latent TB in adults and children (Narayanan 2006; ICMR 1999). Due to the lack of efficacy (0 – 80 % for pulmonary TB (Colditz et al. 1994)) and an associated risk of infection with the viable vaccine in TB-HIV population (Reyn et al. 2010) innovative approaches are being pursued to further improve the existing vaccine, as well as to discover new ones (Ottenhoff et al. 2012; Cayabyab et al. 2012; Evans et al. 2013).

2.1.4 TB treatment

Tuberculosis is the leading cause of death among HIV-infected people in developing countries, and treatment is complex due to interactions between anti-retrovirals and anti-TB drugs and increased risk of adverse effects (Lienhardt *et al.*, 2012). In HIV-negative patients the globally accepted standard treatment consists of a four drug chemotherapy over a duration of six months (rifampicin, isoniazid, ethambutol, and pyrazinamide for 2 months, followed by rifampicin and isoniazid for 4 months) with a cure rate of ~ 90 % (WHO, 2010b). The currently used anti-TB drugs have been discovered before 1970 and resistance to all of these pharmaceuticals by bacterial chromosomal

mutations has appeared (Mitchison and Davies, 2012). In 2008, 3.6 % of all TB incidents globally were estimated to involve multi drug resistant TB (MDR-TB, defined as resistance to at least rifampicin and isoniazid). From those MDR-TB cases approximately 5.4 % were found to have extensively drug resistant TB (XDR-TB, defined as MDR-TB plus resistance to a fluoroquinolone and at least one second-line injectable agent, i.e. amikacin, kanamycin, and/or capreomycin) (WHO, 2010a). Even though XDR-mortality has been reduced from 100 to 45 %, it remains a challenge to treat due to the long medication duration (up to 22 months) and the unfavourable side effects of the used substances (Janssen *et al.*, 2012).

2.1.5 TB diagnosis

Only 66 % of the estimated TB-cases worldwide are correctly diagnosed, even less in high-TB-burden, low-resource settings (WHO, 2012). The first step of TB diagnosis is a proper anamnesis, usually followed by imaging techniques such as chest X-ray or computer tomography and microbiological methods (Norbis *et al.*, 2013). The gold standard in TB diagnosis remains the preparation of liquid cultures in selective media from sputum or tissue/body fluid specimens (Kumar and Robbins, 2007), followed by further Mtb specific tests (i.e. MPT64 antigen detection (Kumar *et al.*, 2011)) or sequencing of Mtb genomic DNA. But the cultivation of Mtb is time consuming and not always successful. A variety of nucleic acid amplification tests (NAAT) are commercially available for laboratory-based diagnosis of active TB (i.e. Xpert MTB/RIF (Helb *et al.*, 2010)). These tests allow sensitive and specific detection of Mtb and rifampicin or isoniazid drug resistance. Yet they need special trained stuff, expensive equipment and are prone to intralaboratory DNA contamination (Dheda *et al.*, 2013). Analysis of cell wall lipids by high performance liquid chromatography (HPLC), mass spectroscopy (MS) or gas chromatography (GC) are promising but in need of expensive equipment and highly trained staff (Lefmann *et al.*, 2004).

A great number of TB tests targeting the human immune response to Mtb are commercially available. Interferon gamma release assays (IGRA) use the Mtb-derived antigens ESAT-6 and CFP-10 to stimulate pre-primed T cells in full blood to release interferon gamma (Tsiouris *et al.*, 2006). These tests lack sensitivity and cannot differentiate between TB and LTBI (Brock *et al.*, 2006). For the tuberculin skin test (TST) a patient is intradermally challenged with an extract containing Mtb antigens (purified protein derivative (PPD)), the resulting induration in the skin, which is due to the development of a delayed-type hypersensitivity reaction, is measured in millimetres (Dacso, 1990). This tests are still widely practised in endemic countries, although they give false positive results for BCG vaccinated (Pai *et al.*, 2006) and false negative results

in immune deficient people (Fisk *et al.*, 2003). Serological TB-assays are strongly represented, measuring antibodies against Mtb-related antigens in blood via ELISA or Lateral Flow Immuno Assays (LFIA). These tests displayed poor sensitivity and specificity in randomized controlled trials (Steingart *et al.*, 2011) and their use has been advised against by WHO (WHO, 2011).

Diagnosis of TB in most low- and middle-income countries continues to rely on sputum smear microscopy for acid-fast bacilli (Ziel-Neelsen stain) (WHO, 2012). This technique detects only 40 – 60 % of pulmonary TB cases, is not able to differentiate between Mtb and other ubiquitous mycobacteria (Rai *et al.*, 2006; van Deun and Portaels, 1998), and is less sensitive in children, in HIV co-infected patients and in patients with extrapulmonary TB (Steingart *et al.*, 2006; Perkins and Cunningham, 2007). Improvement of sputum smear microscopy by fluorescent staining and LED visualization led to ~10 % higher sensitivity (Dheda *et al.*, 2013). An overview of current diagnostic tools for TB and LTBI is given in Table 1.

Table 1: Current diagnostic tools for TB and LTBI.

detection of	TB	LTBI
pathogen	culture	-
	sputum smear microscopy	-
	NAAT	-
	histopathology	-
	LFIA (antigen detection)	-
	DNA sequencing	-
	HPLC, GC, MS	
host response	anamnesis	history of TB contacts
	chest X-ray	chest X-ray (exclusion of PTB)
	TST	TST
	IGRA	IGRA
	serology (LFIA, ELISA)	serology (LFIA, ELISA)

Abbreviations: Nucleic acid amplification techniques (NAAT), lateral flow immuno assay (LFIA), high-performance liquid chromatography (HPLC), gas chromatography (GC), mass spectroscopy (MS), radiography (X-ray), Tuberculin skin test (TST), interferon gamma release assay (IGRA), pulmonary TB (PTB), enzyme linked immuno sorbent assay (ELISA).

Only 15 of the 22 high TB burden countries met the WHO recommendation of having one microscopy centre per 100,000 population in 2011, furthermore among the 36 countries with a high burden of MDR-TB, 19 did not have the recommended capacity of one laboratory to perform culture and drug susceptibility testing per five million population in 2011 (WHO, 2012). Even if a laboratory is within reach, that does not mean that the necessary equipment and infrastructure are continuously available (i.e. lack of reagent supply or constant electricity). Furthermore the know-how for maintenance and resistance to environmental conditions of modern high-tech devices is often critical (Norbis *et al.*, 2013). TB is partly a disease of poverty (Kearney *et al.*, 1993). Although

TB treatment is usually free, sometimes patients are required to pay for diagnostic assays (Aspler *et al.*, 2008). Accuracy, simplicity, affordability and technical robustness are important factors for a point of care (POC) TB-test, but the main advantage is its celerity. Rapid diagnosis allows initiation of treatment while the patient is still accessible (Dheda *et al.*, 2013). This is important because a great portion of patients in high TB burden countries do not return to the health-care facility for the uptake of diagnostic results (Millen *et al.*, 2008).

Direct detection of Mtb antigens in human specimens would allow specific diagnostic of active TB independent from the hosts immune response. Furthermore, the use of antibodies binding Mtb antigens in a lateral flow strip assay would facilitate a rapid POC test in a cost effective, easy-to-use format. By now the only commercially available POC Mtb antigen detection test is the Determine TB-LAM (Alere, Waltham, MA, USA). This LFIA detects a component of the mycobacterial cell wall, lipoarabinomannan (LAM), in urine but has poor sensitivity and specificity (Lawn, 2012).

2.1.6 Immunodominant Mtb antigens

Potential target antigens for POC TB-detection in human samples should be selected by the following criteria: substantial expression by bacteria *in vivo*, presence in the extracellular environment or association with the cell membrane, and resistance to degradation by host enzymes (Flores *et al.*, 2011).

ESAT-6 and CFP-10

Early secretory antigenic target 6 kDa (ESAT-6) and culture filtrate protein 10 kDa (CFP-10) are some of the most abundant culture filtrate proteins of Mtb. The genes encoding these antigens (Rv3874 and Rv3875) are located in a gene cluster, the so called region of difference 1 (RD1), are absent in the vaccine strain *M. bovis* BCG and are co-transcribed (Berthet *et al.*, 1998). *M. bovis* BCG displayed increased virulence when complemented with RD1 in comparison to the parental BCG (Majlessi *et al.*, 2005). Inactivation of Rv3874 and Rv3875 in *M. bovis* resulted in dramatically reduced virulence (Renshaw *et al.*, 2005). ESAT-6 and CFP-10 are secreted by an active process, involving the specialized transport system ESX-1 (Brodin *et al.*, 2004), and form a tight 1:1 complex , which is involved in binding to the cell surface of macrophages and monocyte cells (Renshaw *et al.*, 2002; Renshaw *et al.*, 2005). CFP-10 and ESAT-6 are potent T cell antigens (Arend *et al.*, 2000) and are used in the interferon gamma release assays for TB diagnostic (Tsiouris *et al.*, 2006). The antigen ESAT-6 was demonstrated in human plasma (Mukundan *et al.*, 2012) and cerebrospinal fluid (Song *et al.*, 2013) of TB infected individuals.

AlaDH

AlaDH is a functional L-alanine dehydrogenase encoded by the gene *ald* in *M. tuberculosis* (Andersen *et al.*, 1992), probably involved in alanine biosynthesis (Sassetti *et al.*, 2003). In addition, AlaDH might play a role in cell wall synthesis, because alanine is an important constituent of the mycobacterial peptidoglycan layer (Brennan, 2003; Hutter and Singh, 1999). Therefore, AlaDH detection in human samples may be a potent tool for confirmation of living and replicating mycobacteria and thereby diagnostic of active TB. An *ald* knock-out strain of *M. tuberculosis* was able to grow without alanine or glycine as nitrogen source (Giffin *et al.*, 2012), suggesting a non-essential role of the enzyme. *M. bovis* BCG expressing the *M. tuberculosis* ald showed similar survival in both macrophages and mice, indicating a role in pathogenicity (Scandurra *et al.*, 2006). AlaDH is present early in Mtb cultures but lacks a consensus signal peptide (Andersen *et al.*, 1992). Presence and enzymatic activity were demonstrated only in the cell and on the cell membrane, not in the extracellular medium (Giffin *et al.*, 2012).

LAM

Lipoarabinomannan (LAM) is a component of the mycobacterial cell wall (Besra and Brennan, 1997; Chatterjee *et al.*, 1992). It is a lipoglycan that is characterized by three main components: a mannosylphosphatidylmyoinositol linker, a polysaccharide backbone that consists of a mannan and branched arabinan chains, and a capping motif (Petzold *et al.*, 2005). LAM can influence the phagosome maturation, apoptosis and interferon gamma signal transduction in macrophages (Schlesinger *et al.*, 1994). In addition, LAM disturbs the IL12 cytokine secretion in dendritic cells (Maeda *et al.*, 2003). Numerous studies about the potential role of LAM as an effector in *M. tuberculosis* pathogenicity were published (Hetland *et al.*, 1998; Hamasur *et al.*, 1999; Glickman and Jacobs, Jr., 2001; Brennan, 2003; Flynn and Chan, 2003; Briken *et al.*, 2004; Appelmelk *et al.*, 2008; Mishra *et al.*, 2011). LAM and lipomannan (LM) have a significant impact on the cell wall integrity of mycobacteria, furthermore mutation of LAM or LM can increase β-lactam susceptibility and cause attenuated virulence of *M. tuberculosis* (Fukuda *et al.*, 2013). LAM was detected in human serum (Sada *et al.*, 1992; Sarkar *et al.*, 2012), urine (Achkar *et al.*, 2011; Lawn *et al.*, 2012; Mukundan *et al.*, 2012), sputum (Pereira Arias-Bouda *et al.*, 2000) and cerebrospinal fluid (Mathai *et al.*, 2003).

16 kDa

The 16 kDa antigen (α-crystallin (Acr)/heat shock protein X (HspX)) is the most abundant protein in *M. tuberculosis* during its dormant, non-replicative phase but is not present under conditions of logarithmic growth (Yuan *et al.*, 1996; Hu and Coates, 1999), indicating applicability as a marker for LTBI. HspX was suggested to be associated with the Mtb cell membrane (Lee *et al.*, 1992) and to function as a heat induced chaperone in vitro (Chang *et al.*, 1996). Yuan and colleagues (Yuan *et al.*, 1998) generated an Mtb mutant, replacing the 16 kDa encoding gene *acr* with a hygromycin resistance cassette, which showed impaired growth in macrophages. On the contrary, Hu and co-workers (Hu *et al.*, 2006) found an *acr* deletion mutant to exhibit increased growth in macrophages. These results may be conflictive, but indicate none the less an important role in growth regulation in the host. Humoral immune responses against 16 kDa were measured in various studies (Lee *et al.*, 1992; Imaz *et al.*, 2001; Silva *et al.*, 2003; Demissie *et al.*, 2006; Kaushik *et al.*, 2012), suggesting antigen expression during infection.

85 complex

The 85 complex is a major secretion product of Mtb (Wiker and Harboe, 1992; Sonnenberg and Belisle, 1997) which comprises three variant 85 proteins (A, B and C, 68 – 80 % homology) that are encoded by three different genes (fbpA, fbpB and fbpC) (Wiker *et al.*, 1990). Antigen 85 A, B and C possess mycolyltransferase activity, furthermore they are associated with the formation of the mycobacterial cell wall (Belisle *et al.*, 1997; Kremer *et al.*, 2002; Ronning *et al.*, 2004). Disruption of fbpA resulted in the disability of Mtb to replicate within macrophage like cell lines, indicating a key role in virulence (Armitige *et al.*, 2000). It was shown that 85 complex proteins interact with gelatine-binding sites of human fibronectin, enhancing complement-mediated phagocytosis by macrophages (Abou-Zeid *et al.*, 1988; Abou-Zeid *et al.*, 1991; Bentley-Hibbert *et al.*, 1999). The presence of the 85 complex was demonstrated in human serum (Kashyap *et al.*, 2007; Landowski *et al.*, 2001), urine (Bentley-Hibbert *et al.*, 1999), cerebrospinal fluid (Kashyap *et al.*, 2005) and sputum (Wallis *et al.*, 1998).

2.2 Antibodies

Immune responses, innate or adaptive, are reactions directed against an infection with pathogens (viruses, bacteria, fungi or higher eukaryotic parasites). The innate immunity is amongst others characterized by action as a physical and chemical barrier, recruitment of immune cells to the sites of infection, activation of the complement system and activation of the adaptive immune response. This is a hereditary, non-specific first line of defence, conferring no long lasting immunity. On the contrary, the adaptive immune system is a second line of specific defence (found only in higher vertebrates) generating an immunological memory (Murphy *et al.*, 2008). Immunoglobulins (Ig), likewise called antibodies, are the main part of the humoral component of acquired immunity. Antibodies are secreted by plasma cells, a mature differentiated form of B lymphocytes, generated after antigen contact. Antibodies are combining antigen (*anti*body *gen*erator) recognition through a highly variant aminoterminal (N-terminal) region, with activation of effector mechanisms by the conserved carboxyterminal (C-terminal) region (Dübel and Breitling, 1999). Thereby antibodies structurally connect acceptor and effector functions (like opsonisation, activation of the complement dependent cytotoxicity (CDC) and the antibody dependent cellular cytotoxicity (ADCC)) in one molecule (Descamps *et al.*, 1979).

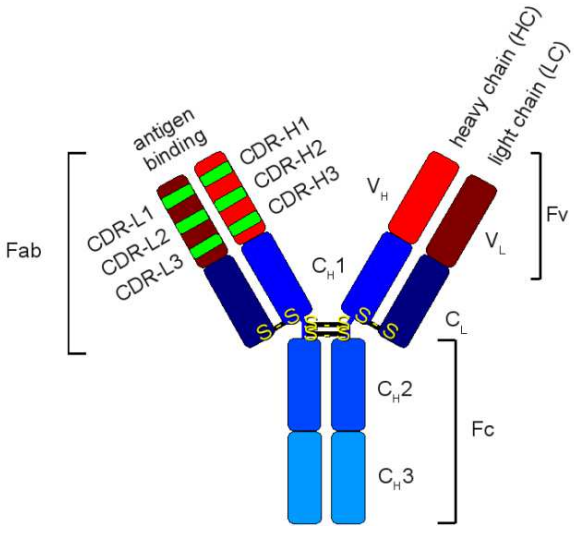


Figure 4: Schematic constitution of an IgG antibody
Adapted from M. Hust with permission. For abbrevations see text.

The antibody matrix is conserved, consisting of four polypeptide chains, stabilized by disulphide and non-covalent bonds between amino acids (Porter, 1973). Two different classes of light chains are known, kappa (κ) and lambda (λ), displaying no difference in function. The effector capacities of an antibody are defined by the constant regions of the heavy chain (α, δ, ε, γ or μ), dividing immunoglobulins into five isotypes (IgA, IgD, IgE,

IgG and IgM) (Abbas and Lichtman, 2011). Affinity matured IgG is the most abundant, providing 80 % of the overall serum immunoglobulin (Murphy *et al.*, 2008). IgG antibodies are combining two identical heavy chains with two identical light chains to a Y-shaped heterotetramer (Harris *et al.*, 1992) (Figure 4). Light chains consist of a variable domain (V_L) at the N-terminus and a constant domain (C_L) at the C-terminus. An IgG heavy chain for example comprises a variable domain at the N-terminus (V_H), followed by a constant domain (C_H1). C_H1 in turn is connected through the *Hinge*-region with the constant domains C_H2 and C_H3. The *Hinge*-region is highly flexible, connecting two heavy chains by disulphide bonds and strong hydrophobic interactions (Davies and Metzger, 1983). Light and heavy chains are connected through disulphide bonds between C_L and C_H1 (Murphy *et al.*, 2008). The better part of the constant antibody domains form the Fc-part (fragment crystallisable), which induces effector functions. The variable areas (Fv, fragment variable) mediate antigen specificity by six hypervariable domains (three per polypeptide chain, consisting of 3 – 20 amino acids). These *complementarity determining regions* (CDR) are flanked by more or less constant *framework* regions (Wu and Kabat, 1970; Griffiths *et al.*, 1993). The six CDRs are in close proximity in a native folded antibody, forming the antigen binding site (paratope), which offers a complementary surface to the antigenic pendant (epitope). The antibody-antigen-binding is conferred through non-covalent interactions like electrostatic attraction, hydrogen bonds, Van-der-Waals forces and hydrophobic interactions (Murphy *et al.*, 2008).

2.2.1 Antibody formats

Different antibody fragments can be obtained by enzymatic cleavage or recombinant methods, which are nowadays preferred due to better handling. In therapeutic applications, small antibody fragments have advantages like better tissue penetration or lack of complement activation (by absence of the Fc-part), but also potential disadvantages like faster renal clearance and reduced stability (Wörn and Plückthun, 2001; Beckman *et al.*, 2007; Schirrmann *et al.*, 2011). The smallest antibody fragment is the *single chain fragment variable* (scFv), which is obtained by genetic fusion of V_H and V_L over a peptide linker (Dübel and Breitling, 1999). The *single chain fragment antigen binding* (scFab) consists of the complete light chain (V_L and C_L) fused to the V_H and C_H1 domains of the heavy chain by a peptide linker (Hust *et al.*, 2007). To overcome problems like fast renal clearance and reduced stability, scFv and scFab can be fused to Fc-parts by a *Hinge*-region, resulting in the so called scFv-Fc or scFab-Fc (scIgG) antibodies (Jäger *et al.*, 2013). Dimerization of two Fc-parts through disulphide bonds leads to bivalent constructs (Powers *et al.*, 2001). These scFv-Fc or scFab-Fc

homodimers possess increased affinity due to an avidity effect. Effector functions like CDC and ADCC are gained, furthermore fusion to effector molecules like toxins or RNases for site directed drug targeting is possible (Kreitman, 2006; Wezler *et al.*, 2012). An overview of the recombinant antibody formats used in this study is given in Figure 5.

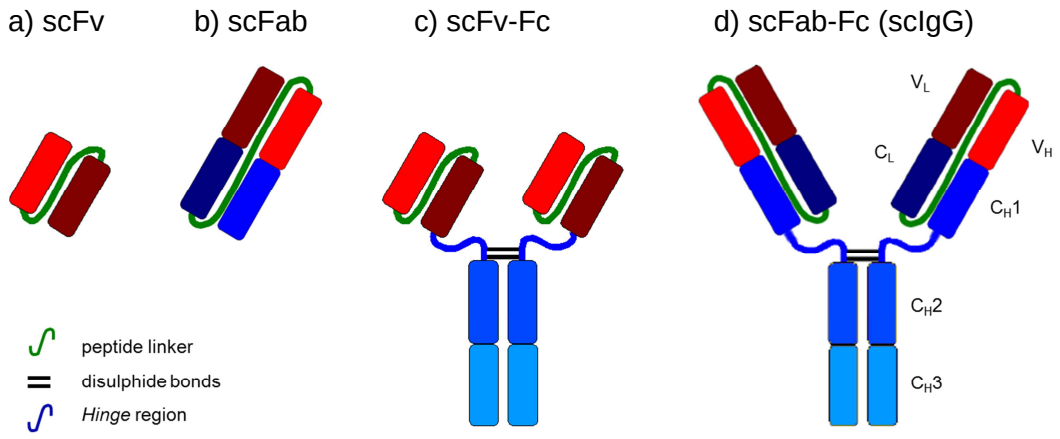


Figure 5: Recombinant antibody formats.
Antibody symbols adapted from M. Hust, with permission.

2.2.2 Generation of recombinant antibodies

Polyclonal antibodies are generated by immunization of animals, a method established for more than a century. The hybridoma technology allows the production of monoclonal antibodies by the fusion of an immortal myeloma cell with an antibody producing spleen cell (Köhler and Milstein, 1975). Both techniques require immunization and are not facilitating the generation of antibodies against conserved mammalian proteins or toxic substances (Winter and Milstein, 1991). Mostly goat, rat, rabbit or mouse antibodies are produced by one of the two methods, which have immunogenic potential, crucial for therapeutic use (Tjandra *et al.*, 1990). The availability of human B-lymphocytes for the generation of human hybridomas is not only limited, but problematical due to ethnical reasons. An alternative is the usage of transgenic animals, but this method is as well limited by the immunization step (Weiner, 2006).

Selection of human antibodies, completely independent from any immune system, is possible by *in vitro* display technologies (Bradbury *et al.*, 2011). Antibody phage display is the most commonly used method, where antibodies are genetically and physically linked to one of the surface proteins (i.e. pIII) of the filamentous bacteriophage M13 (Smith, 1985; Barbas, 3rd *et al.*, 1991). Higher genetic stability and simplification of the antibody gene library amplification was achieved by uncoupling phage propagation from antibody expression (Frenzel *et al.*, 2012). Therefore the antibody: pIII fusion proteins were encoded on a separate plasmid (so called phagemide), containing a phage

morphogenetic signal for packaging the vector into the assembled phage particles (Figure 6). Using the phagemid system, a helper-phage (i.e. Hyperphage (Broders *et al.*, 2003)) is needed for the production of antibody phage particles (Schirrmann *et al.*, 2011).

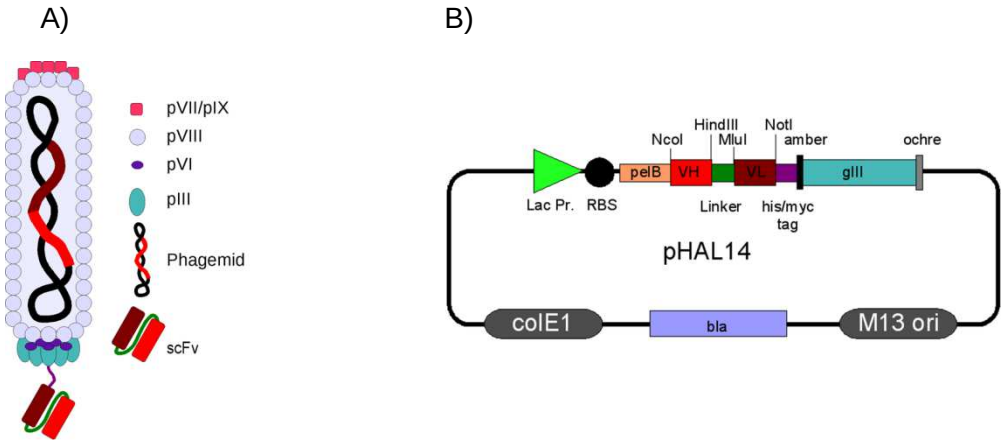


Figure 6: A) Antibody-phage and B) phage display vector pHAL14.
A) Phage presenting a scFv-antibody fused to pIII-protein, pIII-pIX: phage coat proteins, phagemid encoding scFv. B) Phagemid pHAL14, displaying Lac Pr.: lac promotor, RBS: ribosome binding site, VH: antibody heavy chain gene, VL: antibody light chain gene, pelB: secretion signal sequence, bla: beta lactamase gene, gIII: pIII encoding gene, cloning sites, termination sequences and two origins of replication (colE1 and M13 ori). Figure adapted from (Schirrmann and Hust, 2010).

Different types of antibody gene libraries can be constructed in the phage display format. Immune libraries are generated from affinity matured B cells (IgG) of immunized donors, usually to obtain antibodies against a particular pathogenic target (Pelat *et al.*, 2007). Naïve, semi-synthetic and synthetic libraries are constructed to generate antibodies against every possible antigen (Hust and Dübel, 2004; Chan *et al.*, 2011; Tiller *et al.*, 2013). In this study the human naïve libraries HAL4/7/8 are used, constructed from rearranged V genes of B cells (IgM) of non-immunized donors cloned into the phagemid pHAL14 (Figure 6:B) and packaged using Hyperphage (Hust *et al.*, 2011).

2.2.3 Selection of recombinant antibodies by “panning”

The *in vitro* isolation of antibody fragments from antibody gene libraries by their binding activity is called panning. Surface-immobilized antigen is incubated with the respective phage library followed by washing steps to remove unbound phages. After an elution step (i.e. with trypsin), phage are reamplified by infecting *E. coli*. Then the bacteria are infected with a helper-phage to produce new antibody phage. These can be used for further panning rounds until a significant enrichment of antigen specific phage is achieved (Frenzel *et al.*, 2012). Within successive rounds of panning, conditions (i.e. antigen amount, pH, temperature, competition) can be adapted according to the

desired properties (Hoogenboom, 2005). In addition, secondary libraries of selected antibodies can be constructed to further increase the specificity, affinity or stability (Steidl *et al.*, 2008; Thie *et al.*, 2009).

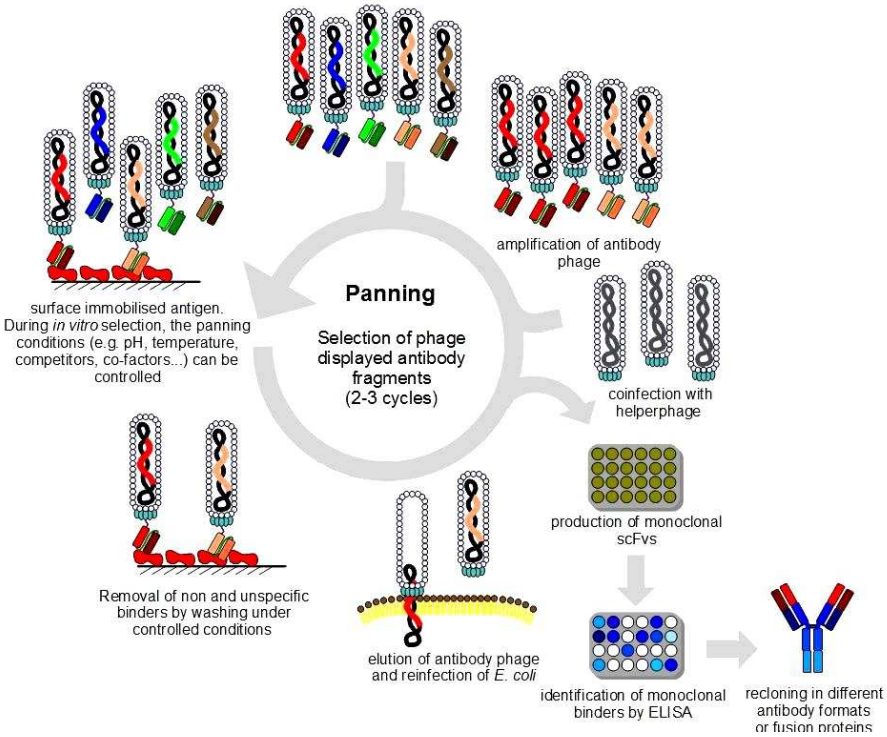


Figure 7: Schematic overview of the selection of antibodies ("panning") by phage display. Figure adopted from (Schirrmann *et al.*, 2011).

2.3 Aim of this work

Tuberculosis is still the leading cause of death due to bacterial infections worldwide (WHO 2012). State of the art TB diagnostic is mostly expensive or time consuming, especially in developing countries a simple point of care TB test is needed (Dheda et al. 2013). Direct detection of Mtb antigens in human specimens would allow specific diagnostic of active TB, independent from the host's immune response. Furthermore, the use of antibodies binding Mtb antigens in a LFIA would facilitate a rapid test in a cost effective, easy-to-use format. For the development of diagnostic assays, recombinant antibodies generated by phage display are an alternative to polyclonal and monoclonal antibodies (Kirsch *et al.*, 2008; Schütte *et al.*, 2009; Meyer *et al.*, 2011; Chan *et al.*, 2013; Lillo *et al.*, 2011). The aim of this work was, first to isolate recombinant antibodies against the Mtb antigens 16 kDa, ESAT-6, CFP-10, LAM, AlaDH, 85 A, 85 B and 85 D from naïve human phage display libraries. Second, a recombinant antibody should be constructed from an IgM expressing hybridome. Third, the generated antibodies ought to be purified, biochemically characterised and examined regarding their suitability in different Mtb antigen detection assays.

3 Materials and Methods

3.1 Materials

3.1.1 Consumables

All consumables used in this study are listed in Table 2.

Table 2: Consumables

product	supplier	catalog number
pipette tips (10 µL)	Biohit, Germany	783211
pipette tips (200 µL)	Biohit, Germany	790201
pipette tips (350 µL)	Biohit, Germany	790350
pipette tips (1000 µL)	Biohit, Germany	791001
pipette tips (1200 µL) extended	Biohit, Germany	791211
polypropylene tubes (15, 50 mL)	Greiner, Germany	188271, 227261
syringes (5, 10, 25 mL)	Braun, Germany	0057.1, 0058.1, 0059.1
syringe filter (0.22 µm)	Millipore, Germany	P668.1
micro centrifuge tubes (1.5 mL)	Greiner, Germany	616201
micro centrifuge tubes (2 mL)	Eppendorf, Germany	10073321
PCR tubes	Abgene, UK	AB-0620
petri dishes	Sarstedt, Germany	82.1473
screw cap tubes (1.5, 2 mL)	Biozym, Germany	710056
PVDF membrane	Millipore, Germany	T831.1
blotting paper	Carl Roth, Germany	4926.1
sensor chip CM5	GE Healthcare, Germany	BR-1000-12
Unisart CN 95 Nitrocellulose membrane	Sartorius, Germany	1UN95ER050025WS
backing cards	DIMA, Germany	custom
cellulose fibre pads	Millipore, Germany	CFSP001750
glass fibre conjugate pads	Millipore, Germany	GFCP000850
LFST cassettes	Shanghai Jieyi Biotechnology, China	custom
Dessicant	Wisepac, UK	MT-1/6
LDPE bags (70 x 100 mm)	Viking, Germany	Q87-B221
screen tape	Agilent, Germany	5067-5371
Microlon 96 Well Plates	Greiner, Germany	655061
Maxisorp Polystyrol 96 Well Plates	Nunc, USA	44-2404-21
PP- Mikrotiterplatten	Greiner, Germany	650201
cover for MTP	Greiner, Germany	656190
seal breathable for MTP	Greiner, Germany	676001
75 cm^2 cell culture flasks	Corning, Netherlands	CLS3290
Vivaspin20 concentrator (MWCO 10 kDa)	Sartorius, Germany	VS2001
Vivacell100 concentrator (MWCO 10 kDa)	Sartorius, Germany	VC1002
polycarbonate Erlenmeyer flasks	Corning, Netherlands	CLS430421
ventilation membrane caps	Corning, Netherlands	CLS431449-4EA
Bio-Scale Mini UNOsphere SUPrA Cartridges	Bio-Rad, Germany	732-4400
Ni-NTA Superflow	QIAGEN, Germany	30450
Sephadex G-25	GE Healthcare, Germany	G25150-500G
glassware	Schott, Germany	

3.1.2 Technical Equipment

The technical equipment used in this study is listed in Table 3

Table 3: Technical equipment

product class	specific type	supplier
0.5 – 10 µL pipette	Reference	Eppendorf
2 – 20 µL pipette	Pipetman	Gilson
10 – 100 µL pipette	Proline Plus	Biohit
20 – 200 µL pipette	Pipetman	Gilson
50 – 1000 µL pipette	Pipetman	Gilson
50 – 300 µL multichannel pipette	Proline Plus	Biohit
50 – 1200 µL eletronic multichannel pipette	eLine	Biohit
pipette controler	pipetus	Hirschmann
analytical balance	EW600-2M	Kern
semi dry electro blotter	Perfect Blue Sedec M	Agilent (Peqlab)
centrifuge (Eppenedorf Cap)	Eppendorf 5414 D	Eppendorf
centrifuge (Falcon tube)	Eppendorf 5810 R	Eppendorf
centrifuge (bottle, SS34)	Sorvall RC5C	Sorvall
Oak Ridge Centrifuge Tube, PC, 50 mL	525-2234	Nalgene
centrifuge bottle with sealing cap, PPCO, 500 mL	3141-0500	Nalgene
clean bench	Hera safe	Heaeus
incubator	Heraeus B12	Heraeus
thermo shaker	PST-60HL-4	lab4you
mini rocker	MR-1	biolab
agarose gel electrophoresis chamber	HW10	Biometra
polyacrylamid gel electrophoresis chamber	Mini Hoeffer SE250	Hoeffer
columns for Ni-NTA, Sephadex	XK-16, XK-26	GE Healthcare
automatic protein purification automate	Profinia 2.0	Biorad
protein purification system	Smartline preparative pump 1800	Knauer
	Smartline UV detector 2520	Knauer
	Smartline 2900 conductivity/pH monitor	Knauer
	Dynamic mixing chamber	Knauer
	Foxy R1 fraction collector	Teledyne Isco
HPLC system	PLATINblue HPLC Plus system	Knauer
ELISA washer	Ultrawash 96 channel	Dynatec
ELISA reader	MRX	Dynatec
thermocycler	Tpersonal	Biometra
surface plasmon resonance analysis unit	Biacore 2000	Biacore
Tape Station System	2200	Agilent (Peqlab)
Milli-Q ultrapure water facility	07.4415	TKA
dispenser	xyz-dispenser	Biodot
guillotine-cutter	CM4000	Biodot
gel documentation station	Herolab Easy	LTF
Transilluminator	Dark Reader DR89X	Clare Chemical
autoclave	Fedegari Autoclavi Spa	Integra
spectrophotometer	Nanophotometer	Implen

3.1.3 Chemicals, buffers and solutions

All chemicals used were p.a. purity grade and purchased from Sigma, Merck or Carl Roth (all located in Germany) or as indicated otherwise. All buffers and solutions used in this study are listed in Table 4. They were either prepared with Milli-Q ultra pure water [0.055 mS cm^{-1}] or as indicated otherwise.

Table 4: Buffers and solutions

purpose	solution	recipe
ELISA	PBS (Sambrook and Russell, 2001)	137 mM NaCl, 2.5 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4
	PBS-B	PBS + 1 % (w/v) BSA
	PBST0.1	PBS + 0.1 % (w/v) Tween-20
	PBST0.05	PBS + 0.05 % (w/v) Tween-20
	PBST-B	PBST0.1 + 1 % (w/v) BSA
	MPBST	PBST0.1 + 2 % (w/v) skim milk
	TMB (3,3',5,5'-tetramethylbenzidine)	ready to use (Seramun, S-004-5-TMB)
	stop solution	0.2 M H_2SO_4
panning	panning block	PBS + 1 % BSA + 1 % skimmed milk
	trypsin	10 µg/mL in PBS
preparation of the periplasmatic fraction	PE buffer	20 % (w/v) sucrose; 50 mM Tris/HCl; 1 mM EDTA; pH 8.0
preparation of the osmotic shock fraction	OS buffer	5 mM $MgSO_4$
epitope mapping	10x PBS	1.5 M NaCl, 92 mM Na_2HPO_4, 16 mM NaH_2PO_4, pH 7.2
	TBS	6.06 g tris base, 0.2 g KCl, 8 g NaCl, pH 8.0, ad 1 L
	TBS-T	TBS + 0.1 % (w/v) Tween-20
	regeneration buffer	62.5 mM tris, 2 % (w/v) SDS, 100 mM ß-mercaptoethanol, pH 6.7
SDS-PAGE	anode buffer	200 mM tris base, pH 8.9
	cathode buffer	100 mM tris base, 100 mM tricine, 0.1 % (w/v) SDS, pH 8.25
	Rotiphorese Gel 40 (ready to use, Roth, A515.1)	30 % (w/v) acrylamide, 0.8 % (w/v) bis-acrylamide
	ammonium persulfate (APS)	40 % (w/v)
	gel buffer	3 M tris base, 0.3 % (w/v) SDS, pH 8.45
	bromophenol blue	0.1 % (w/v)
	2x sample buffer	100 mM tris/HCl, 200 mM DTT, 4 % (w/v) SDS, 0.2 % (w/v) bromophenol blue, 20 % (w/v) glycerol, pH 6.8
	Page Ruler Unstained Protein Ladder	ready to use (Fermentas, SM0661)
	Page Ruler Prestained Protein Ladder	ready to use (Fermentas, SM0671)
western blot	transfer buffer	60 mM tris, 48.8 mM Glycin, 0.46 % (w/v) SDS, pH 8.3
immunostain	TMB membrane substrate	ready to use (Seramun, S-002-4-TMB)
coomassie staining	staining	40 % (v/v) ethanol, 10 % (v/v) acetic acid, 1.45 mM coomassie

		brilliant blue
	destaining	25 % (v/v) ethanol, 8 % (v/v) acetic acid
silver staining	fixation	20 % (v/v) ethanol 12 % (v/v) acetic acid 0.5 mL/L formaldehyde (37 %)
	dehydration 1	50 % (v/v) ethanol
	dehydration 2	30 % (v/v) ethanol
	pre-treatment	0.2 g/L $Na_2S_2O_3$
	staining	2 g/L $AgNO_3$ 0.75 ml/L formaldehyde (37 %)
	development	60 g/L Na_2CO_3 4 mg/L $Na_2S_2O_3$ 0.5 mL/L formaldehyde (37 %)
	stop	10 % (v/v) ethanol 12 % (v/v) acetic acid
Biacore	EDC	75 g/L
	NHS	11.5 g/L
	ethanolamine	1 M
	acetate buffer	10 mM sodium acetate pH 4.0
	$MgCl_2$	3 M
Ni-NTA affinity chromatography	running buffer	20 mM tris/HCl, 500 mM NaCl, pH 8.0
	elution buffer	20 mM tris/HCl, 500 mM NaCl, 500 mM imidazole pH 8.0
Profinia protein purification	running buffer	20 mM Na_2HPO_4, 20 mM NaH_2PO_4, pH 7.2
	elution buffer	100 mM trisodiumcitrate, pH 3.0
	neutralization buffer	1 M tris/HCl, pH 9.0
analytical SEC	running buffer	10 mM NH_4HCO_3 pH 8.0 or PBS pH 7.4
PCR	dNTP	dATP, dCTP,d GTP, dTTP (10 mM each) ready to use (Fermentas, R0191)
agarose gel electrophoresis	TBE	89 mM tris base, 89 mM boric acid, 2 mM EDTA pH 8.0
	6x sample buffer	0.5x TBE, 43.5 % (w/v) glycerol, 0.1 % (w/v) bromphenol blue, 0.1 % (w/v) xylenecyanol blue
	100 bp DNA Ladder	ready to use (Fermentas, SM0243)
	1 kb DNA Ladder	ready to use (Fermentas, SM0313)
DNA staining	Gel Star Nucleic Acid Stain	1:40,000 in TBE (Lonza, 50535)
DNA storage	EB (elution buffer)	10 mM Tris/HCl, pH 8.5

3.1.4 Enzymes

All enzymes used in this study are listed in Table 5.

Table 5: Enzymes and appropriate buffers

enzyme	supplier	catalog number
restriction endonuclease NcoI (10 U/µL)	Fermentas, Germany	ER0571
restriction endonuclease NotI (10 U/µL)	Fermentas, Germany	ER0591
restriction endonuclease HindIII, HC (50 U/µL)	Fermentas, Germany	ER0503

restriction endonuclease NdeI (10 U/µL)	Fermentas, Germany	FD0584
restriction endonuclease NheI (10 U/µL)	Fermentas, Germany	ER0971
restriction endonuclease MfeI (10 U/µL)	Fermentas, Germany	ER0751
restriction endonuclease XhoI (10 U/µL)	Fermentas, Germany	FD0694
restriction endonuclease MluI (10 U/µL)	Fermentas, Germany	ER0561
restriction endonuclease buffer O	Fermentas, Germany	included with enzyme
restriction endonuclease buffer Tango	Fermentas, Germany	included with enzyme
restriction endonuclease buffer R	Fermentas, Germany	included with enzyme
restriction endonuclease buffer EcoRI	Fermentas, Germany	included with enzyme
T4 DNA Ligase (3 U/µL)	Promega, Germany	M180A
T4 DNA Ligase buffer	Promega, Germany	included with enzyme
Superscript Reverse Transcriptase II	Invitrogen, Germany	18064-022
5 x RT-Puffer	Invitrogen, Germany	included with enzyme
Phusion® High-Fidelity DNA Polymerase (2u/µl)	Finnzymes, Keilaranta	F530L
Phusion-Puffer HF 5x	Finnzymes, Keilaranta	included with enzyme
DreamTaq™ Green PCR Master Mix (2X)	Fermentas, Germany	K1081
RNaseOut	Invitrogen, Germany	VX10777019

3.1.5 Antibodies and antigens

All antibodies used in this study are listed in Table 6

Table 6: Commercial and in-house produced antibodies

antibody	format	conc. [mg/mL]	antibody type	supplier (catalog number)	dilution WB / ELISA
Mouse α-C-Myc-tag (9E10)	IgG		monoclonal	in-house produced culture supernatant	1:2,000 / 1:1,000
Mouse α-C-Myc-tag-HRP (9E10)	IgG	1.00	monoclonal	Abcam (ab62928)	1:10,000 / -
Goat α-mouse IgG (Fc specific)-HRP	IgG	6.00	polyclonal	Sigma (A0168)	1:12,000 / 1:30,000
Goat α-mouse IgG (Fab specific)-HRP	IgG	5.70	polyclonal	Sigma (A2304)	- / 1:20,000
Goat α-human-IgG (Fc specific)-HRP	IgG	7.40	polyclonal	Sigma (A0170)	1:10,000 / 1:130,000
Goat α-mouse-IgM (µ specific)-HRP	IgG	3.00	polyclonal	Sigma (A8786)	1:10,000 / 1:2,000
Mouse α-LAM (Lx143)	IgM	1.06	monoclonal	Lionex (Lx143)	- / 1:1,000
Mouse α-penta-His-tag	IgG	0.10	monoclonal	QIAgen (34660)	1:20,000

All antigens used in this study are listed in Table 7.

Table 7: Antigens

antigen	Uniprot ID	origin	used batches	provider
Mtb LAM	-	purified from *M. tuberculosis* H37Rv extract	09-2/1	Lionex
BCG LAM	-	purified from *M. bovis* BCG extract	11-1/1	Lionex
AlaDH	P30234	recombinant *M. tuberculosis* gene Rv2780 expressed in and purified from *E. coli*	09-1/1, 08-1/1	Lionex
ESAT-6	P0A564	recombinant *M. tuberculosis* gene Rv3875 expressed in and purified from *E. coli*	06-1/1	Lionex
CFP-10	POA566	recombinant *M. tuberculosis* gene Rv3874 expressed in and purified from *E. coli*	09-1/1	Lionex
85 A (FbpA)	P0A4V2	recombinant *M. tuberculosis* gene Rv3804c expressed in and purified from *E. coli*	07-1/1	Lionex
85 B (FbpB)	P0C5B9	recombinant *M. tuberculosis* gene Rv1886c expressed in and purified from *E. coli*	10-1/1, 10-1/2	Lionex
85 D (FbpD)	P0A4V6	recombinant *M. tuberculosis* gene Rv3803c expressed in and purified from *E. coli*	07-1/3	Lionex
16 kDa	B2I424	recombinant *M. tuberculosis* gene Rv2031c expressed in and purified from *E. coli*	04-3/1, 04-3/2, 12-2/1, C11-1/1	Lionex

3.1.6 Commercial kits

All commercially available kits used in this study are listed in Table 8.

Table 8: Commercial kits

purpose	specific type	supplier	catalog number
plasmid DNA isolation	QIAprep Spin Miniprep Kit	QIAGEN, Germany	27106
	QIAGEN Plasmid Midi Kit	QIAGEN, Germany	12143
DNA purification	QIAquick Gel Extraction Kit	QIAGEN, Germany	28706
	QIAquick PCR Purification Kit	QIAGEN, Germany	28104
reducing gel analysis of proteins	Screen Tape P200 Protein Standard Kit	Agilent, Germany	5067-5371
RNA isolation	RNeasy Mini Kit	QIAGEN, Germany	74104
coupling HRP to proteins	EZ-Link Plus Activated Peroxidase Kit	Pierce, Germany	31489
T/A-cloning	TOPO T/A-cloning Kit	Invitrogen, Germany	450641
reverse transcription of RNA to cDNA	Superscript II Kit	Invitrogen, Germany	18064-022
protein determination	DC Protein Assay I	Bio-Rad, Germany	500-0111
surface plasmon resonance scFv-Fc	Human Antibody Capture Kit	GE Healthcare, Germany	BR-1008-39

3.1.7 Materials for cultivation and storage of organisms

All media were prepared with deionized water (dH_2O) or as indicated otherwise.

3.1.7.1 Prokaryotes

All media and supplements used for cultivation of *E. coli* are listed in Table 9.

Table 9: Media and supplements used for cultivation of *E. coli*

purpose	medium/supplement	recipe
standard culture	2xYT (Sambrook and Russell, 2001)	1.0% (w/v) Bacto yeast extract, 1.6% (w/v) Bacto tryptone, 0.5% (w/v) NaCl, pH 7.0
	2xYT-A	2xYT + 100 µg/mL ampicillin
	2xYT-G/A	2xYT + 100 µg/mL ampicillin + 100 mM glucose
	ampicillin stock solution	100 mg/mL in Milli-Q
	glucose stock solution	2 M in Milli-Q
panning	2xYT-A/K	2xYT + 100 µg/mL ampicillin + 10 µg/mL kanamycin
	2xYT-T	2xYT + 5 µg/mL tetracyclin
	2xYT-G/A agar	2xYT-G/A + 15 g/L agar-agar
	kanamycin stock solution	10 mg/mL in Milli-Q
	tetracyclin stock solution	5 mg/mL in 96 % (v/v) ethanol
chemically competent cells	TFB1	50 mM $MnCl_2 \cdot 2\ H_2O$, 100 mM RbCl, 50 mM $MgCl_2$, 30 mM CH_3COOK, 10 mM $CaCl_2$, 15 % (w/v) glycerol, pH 5.8
	TFB2	10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2$, 15 % (w/v) glycerol, pH 8.0
transformation	SOC	0.5% (w/v) Bacto yeast extract, 2.0% (w/v) Bacto tryptone, 0.05% (w/v) NaCl pH 7.0, after autoclaving addition of 20 mM $MgCl_2$, 20 mM glucose
expression	2xYT-S/A-IPTG	2xYT + 50 mM sucrose + 100 µg/mL ampicillin + 50 µM IPTG
	sucrose stock solution	1 M in Milli-Q
	IPTG stock solution	50 mM in Milli-Q
	TB (Sambrook and Russell, 2001)	12 % (w/v) Bacto tryptone, 24 % (w/v) yeast extract, 4.3 % (w/v) glycerol ad 900 mL
	TB-salts	0.17 M KH_2PO_4, 0.72 M K_2HPO_4, 100 mL
	TB-G/A	TB + 100 µg/mL ampicillin + 100 mM glucose
storage	2xYT-G/A + glycerol	2xYT-G/A + 30 % (w/v) glycerol

All media and supplements used for cultivation of *M. tuberculosis* are listed in Table 10.

Table 10: Media and solutions used for cultivation of *M. tuberculosis*

purpose	medium/supplement	recipe
standard culture	Difco™ Middlebrook 7H9 Broth (dehydrated culture media, BD, 271310)	approximate formula per 900 mL (supplier declaration): Ammonium Sulfate 0.5 g, L-Glutamic Acid 0.5 g, Sodium Citrate 0.1 g, Pyridoxine 1.0 mg, Biotin 0.5 mg, Disodium Phosphate 2.5 g, Monopotassium Phosphate 1.0 g, Ferric Ammonium Citrate 0.04 g, Magnesium Sulfate 0.05 g, Calcium Chloride 0.5 mg, Zinc Sulfate, Copper Sulfate 1.0 mg, ad 900 mL, pH 6.6 ± 0.2
	BBL™ Middlebrook ADC Enrichment (BD, 211887)	Approximate formula per 1000 mL (supplier declaration): Sodium Chloride 8.5 g, Bovine Albumin (Fraction V) 50.0 g, Dextrose 20.0 g, Catalase 0.03 g, pH 6.9 ± 0.2
	7H9 + ADC + Tween	900 mL Difco™ Middlebrook 7H9 Broth + 100 mL BBL™ Middlebrook ADC Enrichment + 0.05 % (w/v) Tween-80
	Tween-80 stock solution	1 % (w/v)Tween-80 in Milli-Q
	7H9 + ADC + Tween + Gm + Cm + E*	7H9 + ADC + Tween + 80 µg/mL gentamycin + 34 µg/mL chloramphenicol + 10 µg/mL ethambutol
	ethambutol stock solution	10 mg/mL ethambutol-dihydrochloride in methanol
	gentamycin stock solution	80 mg/mL gentamycin sulfate in Milli-Q
	chloramphenicol stock solution	34 mg/mL in 96 % (v/v) ethanol
concentration of culture filtrate	Sauton's	4 g/L L-asparagine x H_2O, 0.5 g/L $MgSO_4$ x 7 H_2O, 2 g/L citric acid x H_2O, 0.66 g/L K_2H PO_4 x 3 H_2O, 0.05 g/L ammonium iron (III) citrate, 4.83 g/L glucose x H_2O, 4.83 g/L pyruvic acid sodium salt, 73.8 g/L 87% (w/v) glycerol, pH 6.8
	Sauton's + Gm +Cm + E*	Sauton's + 80 µg/mL gentamycin + 34 µg/mL chloramphenicol + 10 µg/mL ethambutol

*Addition of antibiotics after sterile filtration of culture fluid.

3.1.7.2 Eukaryotes

All media, solutions and supplements used for culture of mammalian cells are listed in Table 11.

Table 11: Media, solutions and supplements used for cultivation of mammalian cells

purpose	medium/supplement	working concentration
suspension culture of HEK293-6E	FreeStyle F17 medium (Invitrogen, 10388033)	
	G418 sulfate (50 µg/mL, Invitrogen, 10131-027)	25 mg L^{-1}
	pluronic F68 (Applichem, A1288,0100)	1 g L^{-1}
	L-glutamine (PAA, M11-004)	4 mM
	tryptone N1 (19553, Organotechnie S.A.S., France)	0.5 % (w/v)
transfection of HEK293-6E cells	polyethyleneimide (Polysciences, Germany)	2.5 µg mL^{-1}
harvesting of culture supernatant from HEK293-6E cells	Fetal calf serum (FCS) (Invitrogen, 10084-168)	1 % (v/v)
	PMSF (Carl Roth, 6367.1)	1 mM
adherent culture of hybridoma Lx143	DMEM high glucose with L-glutamine and sodium pyruvate (GE Healthcare, E15-843)	
	FCS	10 % (w/v)
	Penicillin-Streptomycin 5000 U mL^{-1} (Pen/Strep, PAA, P11-010)	1 % (v/v)

3.1.8 Organisms

3.1.8.1 Bacterial strains and bacteriophages

All bacterial strains and bacteriophages used in this study are listed in Table 12.

Table 12: Bacterial strains and bacteriophages

strain	genotype	origin
***E. coli* XL1 Blue MRF'**	supE44 hsdR17 recA1 endA1 gyrA46 thi relA1lac-F' [proAB + lacq ZDM15 TN10(tetR)]	Stratagene, Germany
helper phage M13K07	-	Agilent (Vieira and Messing, 1987)
***M. tuberculosis* H37Rv**	-	Lionex

3.1.8.2 Eukaryotic cell lines

All eukaryotic cell lines used in this study are listed in Table 13.

Table 13: Eukaryotic cell lines

strain	description	origin
HEK293-6E	Immortalized human embryonic kidney cells, expression of Epstein-Barr-Virus nuclear antigen 1, suspension culture	NRC, BRI, Canada
Lx143	mouse hybridome expressing α-LAM IgM, suspension culture	Lionex

3.1.9 Molecular vectors

All molecular vectors used in this study are listed in Table 14.

Table 14 Molecular vectors

purpose	vector	description	supplier / origin
antibody sequence isolation	pCR2.1-TOPO	prokaryotic subcloning vector (heavy or light chain only)	Invitrogen
antibody library, phage display	pHAL14	phagemid vector (scFv-pIII)	TU-BS (Hust *et al.*, 2011)
antibody library, phage display	pHAL20	phagemid vector (scFab-pIII)	TU-BS (M. Hust unpublished)
expression in *E. coli*	pOPE101-XP	prokaryotic expression vector (scFv, scFab)	TU-BS (Hust *et al.*, 2009)
expression in HEK293-6E	pCSE2.5-hIgG1-Fc-XP	eukaryotic transient expression vector (scFv-Fc, scFab-Fc)	TU-BS (Jäger *et al.*, 2013)

3.1.10 Oligonucleotides

All oligonucleotides used in this study were synthesized by Metabion Germany or as indicated otherwise and are listed in Table 15.

Table 15: Oligonucleotides

name	sequence (5' – 3')	application
Oligo(dT)$_{12-18}$	dPO$_4$ [(T)12-18] (Invitrogen, 18418-012)	cDNA synthesis
Bi3*	GAG GTG AAG CTG CAG GAG TCA GGA CCT AGC CTG GTG	isolation mouse V_H fo
Bi3b*	AGG TSM AAC TGC AGS AGT CWG G	isolation mouse V_H fo
Bi3d*	AGG TSC AGC TGC AGS AGT CWG G	isolation mouse V_H fo
mouse V_H rv*	ATT TGG GAA GGA CTG ACT	isolation mouse IgM V_H C_H1 rv
Bi5*	GGG AAG ATG GAT CCA GTT GGT GCA GCA TCA GC	isolation mouse V_LK rv
Bi6*	GGT GAT ATC GTG ATR ACM CAR GAT GAA CTC TC	isolation mouse V_LK fo
Bi7*	GGT GAT ATC WTG MTG ACC CAA WCT CCA CTC TC	isolation mouse V_LK fo
Bi8*	GGT GAT ATC GTK CTC ACY CAR TCT CCA GCA AT	isolation mouse V_LK fo
NS21	GGT GAY ATY CAR ATG ACN CAR WSN CCN GCN WSN YTN WS	isolation mouse V_LK fo

M13 fwd	GTT TTC CCA GTC ACG AC	TOPO-cloning colony PCR
M13 rev	CAG GAA ACA GCT ATG AC	TOPO-cloning colony PCR
M13-FP	TGT AAA ACG ACG GCC AGT	Sequencing of TOPO-constructs
MFU_LAM_VL_MluI_fo	ACC GCC TCC ACG CGT AGA TAT CGT GAT GAC CCA G	integration of restriction sites to α-LAM V_L
Mouse IgM VL rv NotI	ACC GCC TCC GCG GCC GCA GTT GGT GCA GCA TCA GC	integration of restriction sites to α-LAM V_L
MFU_LAM_VH_NcoI_fo	ACC GCC TCC CCA TGG CCG AGG TGA AGC TGC AG	integration of restriction sites to α-LAM V_H
Mouse IgM VH rv HindIII	GTCCTCGCAAAGCTTATTTGGGAAGGA CTGACT	integration of restriction sites to α-LAM V_H
MHLacZPro_f	CTA AAG TTT TGT CGT CTT TCC	colony PCR pHAL14
MHgIII_r1	GGC TCG TAT GTT GTG TGG	colony PCR pHAL14
MFU a-LAM scFab VLK Fo NcoI	GAG TGA CCA TGG CCG ATA TCG TGA TGA CC	subcloning of α-LAM scFv to scFab
MFU a-LAM scFab VLK Rv XhoI	ACG AGA CTC GAG CTT GGT GCC TCC ACC	subcloning of α-LAM scFv to scFab
MFU a-LAM scFab VH Fo MfeI	GCG AGC CAA TTG CAG GAG TCA GGA CCT	subcloning of α-LAM scFv to scFab
MFU a-LAM scFab VH Rv NheI	GCG AGC GCT AGC TGA GGA GAC GGT GAC	subcloning of α-LAM scFv to scFab
MHCH1_r3	GGT GCT CTT GGA GGA GGG	colony PCR pHAL20
MHpOPE_f2	CTT AGA TTC AAT TGT GAG CGG	colony PCR pOPE101-XP
MHpOPE_r2	CTG ATC ATT AGC ACA GGC C	colony PCR pOPE101-XP
α-LAM VLK Fo mut2 MluI	ACC GCC TCC ACG CGT AGA TGT CGT GAT GAC CCA AAC TC	mutation α-LAM V_Lκ
α -LAM VH Fo mut2 NcoI	ACC GCC TCC CCA TGG CCG AGG TGA AGC TGG AGG AGT CA	mutation α-LAM V_H
α -LAM scFab2 VLK Fo NcoI	AGC GCG TAA CCA TGG CCG ATG TCG TGA TGA CCC AA	integration of restriction sites to α-LAM V_Lκ
α -LAM scFab2 VLK Rv XhoI	ACC GCC TCC CTC GAG CTT GGT GCC TCC ACC GAA	integration of restriction sites to α-LAM V_Lκ
α -LAM scFab2 VH Fo MfeI	ACC GCC TCC CAA TTG GAG GAG TCA GGA GGT GGC	integration of restriction sites to α-LAM V_H
α -LAM scFab2 VH Rv NheI	ACC GCC TCC GCT AGC TGA GGA GAC GGT GAC TGA	integration of restriction sites to α-LAM V_H
Tor-pCMV-mIgG01-FC-seq-f	CAC TTT GCC TTT CTC TCC	colony PCR pCSE2.5-hIgG1-Fc-XP
Tor-pCMV-mIgG01-Fc-seq-r	CAG ATG GCT GGC AAC TAG	colony PCR pCSE2.5-hIgG1-Fc-XP

*(Dübel *et al.*, 1994)

3.1.11 Peptides

Peptides were N-acetylated and covalently bound to cellulose-β-alanine membranes in form of peptide-spots (~5 nmol per spot). Peptide-spot-membranes were rather purchased at JPT[1] (Berlin, Germany) or provided by Susanne Daenicke[2] (Helmholtz Centre for Infection Research, Braunschweig, Germany) as shown in Table 16. For further details see appendix.

Table 16: Peptide-spot-membranes

antigen	total aa	peptide count	peptide length [aa]	offset [aa]	overlap [aa]	orientation on membrane (lane x spots)
16 kDa[1]	144	34	15	4	11	2 lanes (1x20, 1x14)
85 A[2]	338	109	15	3	12	5 lanes (4x25, 1x9)
85 B[2]	325	105	15	3	12	5 lanes (4x25, 1x5)
CFP-10[2]	100	30	15	3	12	2 lanes (1x25, 1x5)

3.1.12 Colloidal gold- antibody conjugates

Coupling of antibodies to colloidal gold was performed by Susanne Kämpfer (Lionex, Germany).

3.1.13 Software and databases

All software and databases used in this study are listed in Table 17.

Table 17: Software and databases

designation	purpose	reference
BIAevaluation 4.1	analysis of BIAcore results	BIAcore
EMBOSS Transeq	translation of nucleic acid sequences in corresponding aminoacid sequences	(Rice *et al.*, 2000)
ExPASy Protparam	computation of physical and chemical parameters for a protein	http://web.expasy.org/protparam/ (Artimo *et al.*, 2012)
Finch TV 1.4.0	display of sequencing data	Geospiza Inc., USA
Vector NTI Advance 10.3	generation of vector maps	InforMax Inc., USA
MultAlign	Multiple sequence alignment with hierarchical clustering	http://bioinfo.genotoul.fr/ multalin/multalin.html (Corpet, 1988)
Protein BLAST	Protein Basic Local Alignment Search Tool	NCBI (National Centre for Biotechnology Information) (Altschul *et al.*, 1990)
PubMed	US National Library of Medicine	NCBI http://www.ncbi.nlm.nih.gov/pubmed/
IMGT	international ImMunoGeneTics information system	http://www.imgt.org/ (Lefranc *et al.*, 1999)
VBASE2	comparison of antibody genes to database	http://www.vbase2.org/vbase2.php (Retter *et al.*, 2004)
IEDB	The Immune Epitope Database	www.iedb.org (Vita *et al.*, 2010)
AbCheck	antibody sequence test	(Martin, 1996)
TubercuList	*M. tuberculosis* H37Rv genome database	http://genolist.pasteur.fr/TubercuList/ (Institut Pasteur, France)
EZChrom Elite	operation software for Knauer purification systems	Knauer
2200 TapeStation Software (A.01.02)	operation software for TapeStation	Agilent Technologies

3.2 Methods of molecular biology

Basic molecular biological methods were performed as described in (Sambrook and Russell, 2001).

3.2.1 Preparation of plasmid DNA

Plasmid DNA was prepared from liquid *E. coli* overnight cultures in 2xYT-G/A using QIAprep Spin Miniprep Kit for subcloning, or QIAGEN Plasmid Midi Kit for transfection of mammalian cells according to the manufacturer's instructions. The DNA was eluted with EB.

3.2.2 Isolation of RNA

Total RNA was isolated from liquid cultures of hybridoma cells (5 x 10^6 cells mL^{-1}) using RNeasy Mini Kit according to the manufacturer's instructions. The RNA was eluted with RNase free water.

3.2.3 cDNA synthesis

Using Oligo$(dT)_{12-18}$ as primer the mRNA contained in total RNA was reverse transcribed to cDNA using the Superscript II Kit according to manufacturer's instructions.

3.2.4 Amplification of DNA by PCR

DNA was amplified using polymerase chain reaction (PCR, (Mullis *et al.*, 1986). DNA sequences to be amplified were determined using flanking complementary oligonucleotides. For corresponding purposes the oligonucleotide combinations, PCR mixtures and PCR programs are described below. The sequences of all used oligonucleotides are listed in Table 15.

3.2.4.1 Amplification of antibody genes

Table 18: Oligonucleotide pairs for the amplification of antibody genes

oligonucleotide pair	amplicon
Bi5 + Bi6	α-LAM V_LK from cDNA
Bi5 + Bi7	α-LAM V_LK from cDNA
Bi5 + Bi8	α-LAM V_LK from cDNA
Bi5 + NS21	α-LAM V_LK from cDNA
mouse V_H rv + Bi3	α-LAM V_H from cDNA
mouse V_H rv + Bi3b	α-LAM V_H from cDNA
mouse V_H rv + Bi3d	α-LAM V_H from cDNA
MFU_LAM_VH_NcoI_fo + Mouse IgM VH rv HindIII	introduction of restriction sites to α-LAM V_H
MFU_LAM_VL_MluII_fo + Mouse IgM VL rv NotI	introduction of restriction sites to α-LAM V_LK
MFU α-LAM scFab VH Fo MfeI + MFU α-LAM scFab VH Rv NheI	subcloning of α-LAM scFv to scFab formate introduction of restriction sites to α-LAM V_H
MFU α-LAM scFab VLK Fo NcoI + MFU α-LAM scFab VLK Rv XhoI	subcloning of α-LAM scFv to scFab formate introduction of restriction sites to α-LAM V_LK

Table 19: General PCR mixture for amplification of DNA with Phusion polymerase

component	amount [µL]
5 x Phusion HF Buffer	10
dNTPs (10 mM each)	1
template (cDNA, plasmid, gene)	0.5 – 1
Primer Fo (10 pmol µL^{-1})	1
Primer Rv (10 pmol µL^{-1})	1
Phusion Polymerase (2 U µL^{-1})	0,5
dH_2O	35.5 – 36
∑	50

Table 20: PCR programs.
A) amplification of heavy and light chain sequences from α-LAM hybridoma cDNA, b) introduction of restriction sites to α-LAM heavy and light chain from TOPO-constructs and c) introduction of restriction sites to α-LAM heavy and light chain for subcloning to pHAL20.

	a)		**b)**		**c)**	
step	temp.	time	temp.	time	temp.	time
1. initial denaturation	94.0 °C	180 s	94.0 °C	180 s	98.0 °C	60 s
2. denaturation	94.0 °C	45 s	94.0 °C	45 s	98.0 °C	20 s
3. annealing	50.7 °C	45 s	60.0 °C	45 s	61.0 °C	20 s
4. elongation	72.0 °C	60 s	72.0 °C	60 s	72.0 °C	20 s
5. final elongation	72.0 °C	300 s	72.0 °C	300 s	72.0 °C	300 s
6. storage	16.0 °C	∞	16.0 °C	∞	16.0 °C	∞
cycles: 2 – 4	30 x		30 x		40 x	

3.2.4.2 Colony PCR

Colony PCR is a method were one single bacterial colony is used as template for amplification. During the initial denaturation step of PCR the bacterial cells are disrupted.

Table 21: Oligonucleotide pairs for colony PCR

oligonucleotide pair	amplicon
MHLacZPro_f + MHgIII_r1	multiple cloning site (MCS) of pHAL14
MHLacZPro_f + MHCH1_r3	MCS of pHAL20
MHpOPE_f2 + MHpOPE_r2	MCS of pOPE101-XP
Tor-pCMV-mIgG01-FC-seq-f + Tor-pCMV-mIgG01-Fc-seq-r	MCS of pCSE2.5-hIgG1-Fc-XP

Table 22: General PCR mixture for colony PCR

component	amount [µL]
Dream Taq Green mix	10
Primer Fo (10 pmol µL^{-1})	0.5
Primer Rv (10 pmol µL^{-1})	0.5
dH_2O	9
∑	20
+ one bacterial colony	

Table 23: PCR programs for colony PCR.

	pHAL14, pOPE101-XP		pHAL20, pCSE2.5-hIgG1-Fc-XP	
step	temp.	time	temp.	time
1. initial denaturation	95.0 °C	180 s	95.0 °C	180 s
2. denaturation	95.0 °C	30 s	95.0 °C	30 s
3. annealing	56.0 °C	30 s	51.0 °C	30 s
4. elongation	72.0 °C	70 s	72.0 °C	100 s
5. final elongation	72.0 °C	300 s	72.0 °C	300 s
6. storage	16.0 °C	∞	16.0 °C	∞
cycles: 2 – 4	30 x		30 x	

3.2.5 Agarose gel electrophoresis

DNA was separated using agarose gel electrophoresis (Lee *et al.*, 2012). Therefore the analysed DNA and a size standard were loaded into pre-cast wells in 1 % (w/v) agarose gels and a current of 100 V (300 mA) was applied. The DNA in the agarose gel was afterwards stained with Gel Star Nucleic Acid Stain in TBE for 45 min at room temperature (RT) on a rocker. After three short washing steps with water the DNA could be detected under UV light (λ = 312 nm) due to the flourescent properties of Gel Star (White *et al.*, 1999).

3.2.6 Purification of DNA

DNA purification directly after PCR (only one amplicon) was performed with QIAquick PCR Purification Kit according to the manufacturer's instructions. The DNA was eluted with 30 µL 3.3 mM Tris/HCl pH 8.5.

After PCR (with several amplicons) or enzymatic digestion DNA was purified from agarose gels. For this purpose DNA was separated on 1 % (w/v) agarose gel and stained as described in chapter 3.2.5. Afterwards the flourescent dye was excited by a blue light transilluminator and the DNA fragments were excised with a scalpel. The DNA was then purified with QIAquick Gel Extraction Kit according to the manufacturer's instructions and eluted with 30 µL 3.3 mM Tris/HCl pH 8.5.

3.2.7 T/A-cloning

V_H and V_L genes amplified from cDNA were purified over agarose gels and ligated into pCR2.1-TOPO using the TOPO T/A-cloning Kit according to the manufacturer's instructions.

3.2.8 Digestion of DNA with restriction endonucleases

Enzymatic DNA digestion was carried out using restriction endonucleases type II purchased from Fermentas (Germany). Reaction buffers and incubation temperatures were chosen according to the manufacturer's instructions. Digestion was done for up to 6

hours at usually 37 °C and was followed by heat inactivation of the restriction enzymes (20 min at 65 °C or 80 °C). A typical restriction mixture is shown in Table 24.

Table 24: Typical mixture for enzymatic digestion of DNA with restriction endonucleases.

component	amount [µL]
restriction endonuclease 1 (10 U µL^{-1})	2
restriction endonuclease 2 (10 U µL^{-1})	2
10 x buffer	5
DNA (i.e. PCR amplicon)	30
dH_2O	11
∑	50

3.2.9 Ligation of DNA

Cohesive ends of two strands of DNA were joined by ligation (Engler, M.J. and Richardson, C.C., 1982) by Promega T4 DNA Ligase at 22 °C for three hours. The composition of the ligation mixture was varying due to the size of vector and insert and was determined according to the manufacturer´s instructions and the following equation:

(1**)** $$\frac{ng\ of\ vector \times kb\ size\ of\ insert}{kb\ size\ of\ vector} \times molar\ ratio\ of\ \frac{insert\ (3)}{vector\ (1)} = ng\ of\ insert$$

3.2.10 Sequencing of DNA

All constructed plasmids were sent for extended hotshot sequencing to Seqlab (Germany). Therefore 600 – 700 ng of plasmid were mixed with 20 pmol of oligonucleotide primer and adjusted with dH_2O to a total volume of 7 µL.

3.3 Microbiological methods

3.3.1 Glycerol stocks

For long-term storage of bacteria, glycerol cultures were used. Therefore, 900 µL overnight cultures of *E. coli* in 2xYT-G/A were mixed with 300 µL of 80 % (w/v) glycerol. The suspensions were stored at -80°C.

3.3.2 Chemically competent *E. coli*

20 mL of 2xYT-T were inoculated from glycerol stock with *E. coli* XL-1 blue MRF' and incubated overnight at 37 °C and 120 rpm. From this overnight culture 350 mL of 2xYT-T were inoculated to an OD_{600nm} of 0.07 and grown at 37 °C and 120 rpm till OD_{600nm} of 0.52 was reached.

The culture was harvested by centrifugation (3,220 x g, 4 min, 4 °C). Supernatants after centrifugation were discarded and the bacterial pellets were resuspended in 30 mL of ice

cold TFB1 buffer. After incubation on ice for 90 min, the cells were again centrifuged (3,220 x g, 4 min, 4 °C). Following centrifugation, supernatants were discarded and the cells were resuspended in 4 mL of ice cold TFB2 buffer. Aliquots of 50 µL in 1.5 mL microcentrifuge tubes were sharp frozen with liquid nitrogen. The competent cells were stored at -80 °C.

3.3.3 Transformation of *E. coli* by heat shock

Chemically competent *E. coli* cells were transformed by heat shock. Therefore 10 – 20 µL of DNA was mixed with 50 µL of competent cells and incubated on ice for 20 min. After heating the cells for 45 sec to 42 °C, they were chilled on ice for 2 min. For regeneration, transformed cells were incubated in 1 mL of SOC medium at 37 °C with moderate shaking for 45 min. The transformed *E. coli* were streaked on 2xYT agar plates with appropriate antibiotics and incubated overnight at 37 °C.

3.3.4 Panning in Microtitre plates (MTP)

The selection of recombinant antibodies was performed according to (Schirrmann and Hust, 2010) with modifications. In short, pannings were performed in 96 well microtitre plates (Microlon, Greiner). For the first panning round ten µg of antigen were coated in PBS pH 7.4 overnight at 4 °C. The antigen-coated wells and wells for the preincubation of the library were blocked with MPBST. 2.5 x 10^{11} phage particles of the human naive antibody gene libraries HAL7 and HAL8 (Hust *et al.*, 2011) were diluted in PBST0.1 with 1 % (w/v) skim milk and 1% (w/v) bovine serum albumin (BSA) and preincubated for one hour. The supernatant, containing the depleted library, was incubated in the antigen-coated wells at RT for 2 hours followed by ten washing steps with PBST. Afterwards, bound scFv phage particles were eluted with 200 µL trypsin solution at 37 °C for 30 min. The supernatant containing eluted scFv phage particles was transferred into a new tube. Ten µL of eluted scFv phage were used for titration as described by (Schirrmann and Hust, 2010). Twenty mL *E. coli* XL1-Blue MRF' culture in the logarithmic growth phase (OD_{600nm} = 0.4 - 0.5) were infected with the remaining scFv-phage at 37 °C for 30 min without shaking. The infected cells were harvested by centrifugation for 10 min at 3,220 x g and the pellet was resuspended in 250 µL 2xYT-G/A medium, plated on a 15 cm 2xTY-G/A agar plate and incubated overnight at 37 °C. Colonies were harvested with 5 mL 2xTY-G/A. Thirty mL of 2xYT-G/A were inoculated with 100 µL of the harvested colony suspension and grown to an OD_{600nm} of 0.4 to 0.5 at 37 °C and 250 rpm. Five mL bacterial suspension (~2.5 x 10^9 bacteria) were infected with 5 x 10^{10} helper phage M13K07, incubated at 37 °C for 30 min without shaking, followed by 30 min at 250 rpm. Infected cells were harvested by centrifugation for 10 min at 3,220 x g and the pellet was resuspended in 30 mL 2xYT-A/K. Antibody phage were produced at 30 °C and 250 rpm

for 16 h. Cells were harvested by centrifugation for 10 min at 3,220 x g. The supernatant containing the antibody phage (~1 x 10^{12} cfu mL^{-1}) was directly used for the next panning round or stored at 4 °C. Two panning rounds followed with three and one µg of immobilized antigen. Only clones from the third panning round were picked for screening.

3.3.5 Production of antibody fragments in *E. coli*

3.3.5.1 Production of scFv in MTP

The identification of monoclonal binders was performed as described in (Hust *et al.*, 2009) with modifications. 96-well polypropylene (PP) MTPs containing 150 µL 2xYT-G/A were inoculated with colonies from the titration plate of the third panning round. MTPs were incubated overnight at 37 °C and 850 rpm in a MTP shaker. A volume of 180 µL 2xYT-G/A in PP-MTP well was inoculated with 10 µL of the overnight culture and grown at 37 °C and 850 rpm for two hours. Bacteria were harvested by centrifugation for 10 min at 3,220 x g and 180 µL supernatant were removed. The pellets were resuspended in 180 µL 2xYT-S/A + IPTG and incubated at 30 °C and 850 rpm overnight. Bacteria were pelleted by centrifugation for 15 min at 3,220 x g and 4 °C. The scFv-containing supernatant was transferred to a new PP-MTP and stored at 4 °C prior to analysis.

3.3.5.2 Production of scFv in shaking flasks

Previous to production in shaking flask scale the scFv encoding sequences were subcloned to the expression vector pOPE101-XP. After subcloning expression and secretion of scFv were tested by small scale production of scFv in MTP followed by SDS-PAGE and immunoblot targeting C-myc-tag. For the preculture 80 mL 2xTB-G/A were inoculated from glycerol stock and incubated at 37 °C and 120 rpm overnight. For the main culture 1 L of TB-G/A in a 2 L shaking flask with baffles was inoculated with the overnight culture to an OD_{600nm} of 0,05 and incubated at 37 °C and 120 rpm till OD_{600nm} of 2.0 was reached. Then the expression of scFv was induced by addition of 50 µm IPTG and 50 mM sucrose. Antibodies were produced at 18 °C overnight (16 – 20 hours). The next day bacterial cells were harvested by centrifugation at 5,000 x g and 4 °C for 15 min. The supernatants were discarded and the bacterial pellets were stored at 4 °C until further use.

3.3.6 Cultivation of *M. tuberculosis*

M. tuberculosis (Mtb) H37Rv cultures were provided by Dr. Wulf Oehlmann (Lionex GmbH) Mtb was cultivated in the L3 facility of the University of Veterinary Medicine Hannover. In general 400 – 700 mL of 7H9 + ADC + Tween or Sauton's medium in a 1 L

Schott bottle were inoculated 1:20 with liquid cultures of Mtb in stationary growth phase and incubated without shaking at 37 °C for one week or up to twelve months.

3.3.7 Concentration of culture filtrates of *M. tuberculosis*

Culture filtrates for concentration were obtained only of Mtb cultivated in Sauton's medium. For that purpose 100 – 300 mL of culture were sterile filtrated through 0.22 µm syringe filter. To preserve sterility 80 µg mL^{-1} gentamycin, 34 µg mL^{-1} chloramphenicol and 10 µg mL^{-1} ethambutol were added. Culture filtrates were concentrated 20 to 30 fold with VivaSpin20 or Vivacell100 (Sartorius, Germany) according to the manufacturer's instructions.

3.4 Biochemical methods

3.4.1 Isolation of the periplasmatic and osmotic shock fraction of *E. coli*

The reducing nature of the cytoplasm in *E. coli* inhibits the formation of intradomain disulfide bridges necessary for the correct folding of scFv (Skerra and Plückthun, 1988). Through the expression vector pOPE101-XP, scFv were fused with the N-terminal leader peptide pelB that directs the recombinant protein towards the SEC protein-translocation pathway (Mori and Ito, 2001) into the periplasm of *E. coli*, where it can be properly folded in an oxidizing environment (Popplewell *et al.*, 2005). In addition, through the gentle removal of the outer membrane of *E. coli*, an antibody preparation not contaminated by cytoplasmic proteins can be obtained (Rouet *et al.*, 2012).

The periplasmic enriched fraction (PPP) and osmotic shock fraction (OSP) of *E. coli* were prepared after the method of (Dübel *et al.*, 1995). Cell pellets after production of scFv in shaking flasks were resuspended with 100 mL of ice cold PE buffer and incubated on ice for 20 min with potent mixing every five minutes. After that the suspension was centrifuged at 27,000 x g and 4 °C for 30 min. The resulting supernatant, the PPP of *E.coli*, was collected. The remaining pellet was resuspended with 100 mL of ice cold OS buffer and incubated on ice for 20 min with potent mixing every five minutes. After this step the suspension was centrifuged at 27,000 x g and 4 °C for 30 min. The resulting supernatant, the OSP of *E.coli*, was collected. Samples of PPP and OSP were taken, before both solutions were merged.

3.4.2 Purification of His tagged scFv via immobilized-metal affinity chromatography (IMAC)

The antibody expression vector pOPE101-XP delivers scFv with a sixfold His tag at the N-terminus of the protein (Hust *et al.*, 2009) allowing enrichment on nickel nitrilo tri acetic acid (Ni-NTA) resin (Block *et al.*, 2009). In this study His- tagged scFv were purified from periplasmatic and osmotic shock fractions of *E. coli* under high salt conditions via 5 mL Ni-NTA Superflow (QIAgen, Germany) in XK-16 colomns (GE Healthcare). Ni- NTA XK-16 columns were prepared according to the manufacturer's instructions. The combined PPP + OSP solutions (see chapter 3.4.1) were adjusted to 20 mM Tris/HCl and 500 mM NaCl with a 80 mM Tris/HCl 2 M NaCl pH 8.0 stock solution. The resulting solution was cleared over a folded filter before it was applied to the Ni-NTA using a Smartline preparative pump 1800 (Knauer) and a dynamic mixing chamber (Knauer). The necessary steps for this purification are summarised in Table 25.

Table 25: Overview purification of scFv via Ni-NTA.

step	volume [mL]	solution
equilibration	30	buffer A*
sample application	270	sample
washing	10	buffer A
linear gradient	25	buffer A mixed with 0 – 50 % (v/v) buffer B (0 – 250 mM imidazole)
elution	10	buffer B*
regeneration	20	20 mM MES, 100 mM NaCl, pH 5.0
storage	10	20 % (V/V) ethanol

* running buffer A: 20 mM Tris/HCl, 500 mM NaCl, pH 8.0; elution buffer B: 20 mM Tris/HCl, 500 mM NaCl, 500 mM imidazol, pH 8.0

The purification was carried out with a constant flow rate of 2 mL min^{-1} and maximal 72 psi. Absorption at 280 nm and conductivity [mV] were plotted with Smartline UV detector 2520 and Smartline 2900 conductivity/pH monitor (both Knauer). 2 mL fractions of the washing step, the linear gradient and the elution were collected using Foxy R1 fraction collector (Teledyne Isco, USA) in 7 mL collection tubes, and were later analysed by SDS-PAGE and Coomassie staining.

3.4.3 Protein quantification

Protein concentrations were determined using folin phenol reagent (Lowry *et al.*, 1951) (Peterson, 1979) using the DC Protein Assay (Bio-Rad, Germany) according to the manufacturer's instructions.

3.4.4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

In this study SDS-PAGE (Laemmli, 1970) altered after (Schägger and Jagow, 1987) was performed. The recipes for separating and stacking gels are listed in Table 26.

Table 26: Recipe for four Schaegger and Jagow gels (4 % stacking and 12 % separating).

step	component	stacking gel	separating gel
1	Milli-Q water	11 mL	5.7 mL
2	gel buffer: tricin	4.65 mL	5.9 mL
3	glycerol 86 % (w/v)	-	2.7 mL
4	0.1 % (w/v) bromophenol blue	150 µL	-
5	Rotiphorese Gel 40	2.4 mL	9.4 mL
6	N,N,N',N'-Tetramethylethylendiamin (TEMED)	15 µL	9 µL
7	40 % (w/v) Ammoniumpersulfat (APS)	38 µL	22 µL
	∑	18 mL	27 mL

The separating gel was poured between glass plates of the Hoeffer Mini dual gel caster (10 x 8 cm, Hoeffer, Germany), followed by the stacking gel. A comb was inserted to obtain lanes. After polymerization the gel was attached to the Hoeffer Mini electrophoresis unit and the chamber was filled with cathode and anode buffer according to the orientation of electrodes. Samples were mixed with 2 x sample buffer for SDS-PAGE and boiled for 10 min at 100 °C. After cooling, 100 mM DTT was added to the samples. Samples and a molecular mass standard (Page Ruler Un-/Prestained Protein Ladder) were applied to the lanes of a PAA-Gel. A current of 40 V (10 mA) was applied for electrophoresis through the stacking gel and after that a current of 100 V (60 mA) was applied for electrophoresis through the separating gel till the dye front ran out.

3.4.5 Coomassie staining

PAA-gels were stained with Coomassie Brilliant Blue in coomassie staining solution for 1.5 h to overnight at 50 °C. The PAA-gel was afterwards destained in coomassie destaining solution at RT till an adequate protein pattern was visible.

3.4.6 Silver staining

PAA-gels were silver-stained according to (Blum *et al.*, 1987) with modifications, in brief summarized in Table 27.

Table 27: Silver staining modified after Blum et al. 1987.

incubation time	solution	recipe
1 – 20 h	fixation	20 % (v/v) ethanol 12 % (v/v) acetic acid 0.5 mL/L formaldehyde (37 %)
2 x 20 min	dehydration 1	50 % (v/v) ethanol
1 x 20 min	dehydration 2	30 % (v/v) ethanol
1 min	pre-treatment	0.2 g/L $Na_2S_2O_3$
3 x 20 sec	dH_2O	
20 min	staining	2 g/L $AgNO_3$ 0.75 ml/L formaldehyde (37 %)
variable	development	60 g/L Na_2CO_3 4 mg/L $Na_2S_2O_3$ 0.5 mL/L formaldehyde (37 %)
15 min	stop	10 % (v/v) ethanol 12 % (v/v) acetic acid

3.4.7 Western blot

A semi-dry Western blot was performed to transfer proteins from PAA-gels onto polyvinylidene fluoride (PVDF) membranes. Therefore a PVDF membrane was activated with 10 mL methanol for 1 min followed by addition of 20 mL transfer buffer. The membrane and the gel were equilibrated with the transfer-methanol mixture for 30 min. After equilibration three pieces of blotting paper were soaked with transfer-methanol mixture and applied on the blotting device, followed first by the membrane and second by the gel, finished by another three pieces of soaked blotting paper. Transfer of the proteins was performed with 3 mA cm^{-2} (20 V max.) for 30 min.

3.4.8 Tape Station analysis

The Tape Station is an automated system for protein quality control from 10 to 200 kDa allowing the determination of protein size, product purity and concentration. Proteins were labelled with a pyrylium fluorescent dye (McNeill et al., 2010; Craig *et al.*, 2005), boiled under reducing conditions, mixed with 10 and 200 kDa in line markers and separated on an acrylamide gel matrix by electrophoresis. The obtained results were visualized as a gel image comparable to a standard SDS-PAGE or as a chromatogram where fluorescence units (FU) are outlined against protein size. The purity of a protein preparation is stated as the percentage of the target peak in the overall integrated area.

Purified antibody solutions were analysed with the Screen Tape P200 Protein Standard Kit (Agilent) under reducing conditions on a 2200 Tape Station system (Agilent) according to the manufacturer's instructions. The "P200 molecular weight standard" (Agilent) was used as a molecular marker.

3.4.9 Conjugation of antibodies to horseradish peroxidase (HRP)

Purified scFv-Fc antibodies were conjugated to HRP over reductive amination (Imagawa, M., et al., 1982) with the EZ-Link Plus Activated Peroxidase Kit (Pierce, Germany) following the protocol at pH 9.4 according to manufacturer's instructions.

3.5 Biophysical methods

3.5.1 Preparative size exclusion chromatographie (SEC)

Preparative SEC (Porath and Flodin, 1959) was performed for desalting/buffer exchange of protein solutions after Ni-NTA purification. Therefore 80 mL Sephadex G-25 fine (GE Healthcare) were prepared in a XK-26 (GE Healthcare) column according to the manufacturer's instructions. Eluate fractions containing target protein after Ni-NTA (verified via SDS-PAGE) were pooled and applied to the Sephadex-G25-XK-26 column

using a Knauer Bioline purification system and a Foxy R1 fraction collector as described before. The buffer exchange was carried out with PBS buffer pH 7.4 at a constant flow rate of 5 mL min^{-1} and maximal 72 psi. Absorption at 280 nm and conductivity [mV] were plotted. Seven mL fractions were collected.

3.5.2 Analytical SEC

Analytical SEC was performed to determine the size and aggregational behaviour of proteins. The Knauer PLATINblue HPLC Plus system with a Superdex 200 10/300 GL column (GE Healthcare, USA) was used according to the manufacturer's instructions. The flow rate was constant at 0.5 mL min^{-1}. 500 µL of sample were applied, running buffer was PBS pH 7.4 (for antibodies) or 10 mM NH_4HCO_3 (for antigens), the absorption at 280 nm and the retention times were plotted. Eight molecular standards (listed in Table 28) were used for calibration of the column.

Table 28 Calibration standards for analytical SEC.
Mixture of HMW+LMW calibration kit (GE Healthcare, USA).

protein	molecular mass [kDa]
Thyroglobulin	669
Apoferritin	443
ß-Amylase	200
Alcohol Dehydrogenase	150
Albumin	66
Carbonic Anhydrase	29
Cytochrome c	12.4
Aprotinin	6.5

3.5.3 Affinity measurement via surface plasmon resonance (SPR)

The affinities of scFv and scFv-Fc antibodies were measured by surface plasmon resonance using a CM5 sensor chip and a Biacore2000 (GE Healthcare, Germany). For affinity measurement of α-16kDa scFv antigen was immobilized on the CM5 chip via amine coupling according to the manufacturer's instructions. 1 µg mL^{-1} antigen was dissolved in 10 mM acetate buffer pH 4.0 and 55.3 response units (RU) were immobilized on the surface. During the measurement a flow rate of 30 µL min^{-1} was maintained and four dilutions of scFv in HBS-EP buffer (125 nM, 250 nm, 500 nm, 2x 1000 nm) were associated for 300 sec and dissociated for 700 sec. Regeneration was performed after each scFv dilution with 10 µL 10 mM glycin pH 1.0. Association and dissociation constants were calculated using the Biaevaluation software (Karlsson *et al.*, 1991) and verified with internal consistency tests (Schuck and Minton, 1996). For the affinity measurement of α-85 B scFv-Fc the human antibody capture kit (GE Healthcare, Germany) was used according to the manufacturer's instructions.

3.6 Immunological methods

3.6.1 Immunostain

Proteins blotted onto PVDF membranes were detected using immunostain. Therefore, following western blotting, free binding sites on the PVDF membrane were blocked with MPBST for minimum 30 min at RT. Subsequently, incubation with protein-specific antibody was carried out in MPBST for a minimum of 60 min at RT. After three washing steps with PBST0.1 for 5 min the blot was incubated with a secondary antibody coupled to HRP in MPBST for at least 45 min at RT. After another three washing steps with PBST0.1 the immunostain was developed with TMB peroxidase membrane substrate (ready to use) until an adequate signal was obtained. Development was stopped by three short washing steps with Milli-Q water.

3.6.2 Epitope mapping

Epitope mapping was performed using the technique of oligo-peptide scanning (Morris, 1996). This technique uses a library of oligo-peptide sequences from overlapping segments of a target protein and tests for their ability to bind the antibody of interest. Peptides were N-acetylated and covalently bound to cellulose-β-alanine membranes in form of peptide-spots (Frank and Overwin, 1996). For further details on the peptide-spot-membranes see chapter 3.1.11 and appendix.

The membranes were rinsed with methanol, washed three times for 5 min with TBS and blocked with TBS-B for 1.5 h at RT. After that the membranes were incubated with an antibody generated against the target antigen in a dilution of 0.67 µg mL^{-1} antibody in 15 mL TBS-B for 3 h at RT. After washing three times for 5 min with TBS the membranes were incubated with a secondary antibody conjugated to HRP (Goat α-human IgG (Fc)-HRP 1:20,000 or mouse α-C-Myc-tag-HRP (9E10) 1:1,000) diluted in 15 mL TBS-B for 1.5 h at RT. After another three washing steps with TBS the immunostain could be detected with TMB peroxidase membrane substrate (ready to use). The development was stopped after 5 – 11 min with Milli-Q water. After scanning of the array the membranes were destained by a short washing step with methanol followed by washing with TBS-T till the spots were colourless (usually 3 h to overnight). Regeneration of the peptide spots was carried out according to regeneration protocol I in the JPT PepSpot Peptides protocol (JPT, Germany). In brief, the membranes were washed 3 x 5 min with Milli-Q at RT, 4 x 30 min with regeneration buffer I at 50 °C, 3 x 20 min with 10x PBS for epitope mapping at RT, 3 x 20 min with TBS-T at RT, 3 x 10 min with TBS at RT.

3.6.3 Enzyme linked immune sorbent assay (ELISA)

In this work different types of ELISA (Engvall and Perlmann, 1971; Yalow and Berson, 1960) were used as specified in the following chapters. The standard operation volume per cavity was 100 µL or as indicated otherwise.

3.6.3.1 Indirect ELISA

Screening ELISA:

96 wells of Greiner Microlon MTP were coated with 100 ng of antigen or BSA as a negative control in PBS buffer overnight at 4 °C. After coating, the wells were blocked with PBST-B for 2 h at RT, followed by three washing steps with PBST0.05. For identification of binders, supernatants containing monoclonal scFv (see chapter 3.3.5.1) were incubated in the antigen coated plates for 1.5 h at RT followed by three PBST0.05 washing cycles. Bound scFv were detected using mouse α-c-Myc-tag 9E10 (1:1,000 in PBST-B; 1.5 h at RT) followed by goat α-mouse IgG (Fc)-HRP (1:30,000 in PBST-B; 45 min at RT). After three washing steps with PBST0.05 the reactions were visualized with TMB as a substrate. The staining reaction was terminated by addition of stop solution. Absorbance at 450 nm (620 nm reference) was measured using MRX ELISA reader (Dynatec, Germany).

Antibody titration ELISA:

Per antibody 24 cavities of Greiner Microlon MTP were coated with 100 ng antigen in PBS at 4 °C overnight with BSA coated cavities as a negative control. After coating, the wells were blocked with PBST-B for 2 h at RT, followed by three washing steps with PBST0.05. Twelve dilutions of antibody in PBST-B were applied in duplicates on the antigen and BSA controls and incubated for 1.5 h at RT. Bound scFv-Fc were detected using goat α-human IgG (Fc)-HRP (1:130,000 in PBST-B; 45 min at RT). The assay was further processed as described above.

Antigen titration ELISA:

Twelve dilutions of antigen in PBS buffer were coated in duplicates to 96 wells of Greiner Microlon MTP at 4 °C overnight with BSA coated cavities as a negative control. After coating, the wells were blocked with PBST-B for 2 h at RT, followed by three washing steps with PBST0.05. Antigen detection was carried out with a concentration of antibody at half maximal saturation (determined by antibody titration ELISA) in PBST-B for 1.5 h at RT followed by three PBST0.05 washing cycles. Bound scFv-Fc were detected using

goat α-human IgG (Fc)-HRP (1:130,000 in PBST-B; 45 min at RT). The assay was further processed as described above.

3.6.3.2 Direct ELISA:

Analyte (antigen or antibody) was coated to the surface of Greiner Microlon 96 Well MTP at various concentrations in PBS buffer overnight at 4 °C. Detection of the analyte was carried out with an antibody (commercial or in house produced scFv-Fc) conjugated to HRP diluted in PBST-B for 1 h at RT. The assay was further processed as described above.

3.6.3.3 Sandwich ELISA

Sandwich antigen titration:

100 ng of capture antibodies were coated to the surface of 96 wells of Greiner Microlon MTP in PBS buffer overnight at 4 °C. After coating, the wells were blocked with PBST-B for 2 h at RT, followed by three washing steps with PBST0.05. Twelve dilutions of antigen in PBST-B were applied in duplicates onto the antibody coated cavities and incubated for 1.5 h at RT, followed by three washing steps with PBST0.05. Detection of bound antigen was performed with HRP conjugated scFv-Fc (exact dilutions described in results) in PBST-B for 1.5 h at RT, followed by three washing steps with PBST0.05. The assay was further processed as described above.

Sandwich sample mensuration:

100 ng of capture antibodies were coated to the surface of 96 wells of Greiner Microlon MTP in PBS buffer overnight at 4 °C. After coating, the wells were blocked with PBST-B for 2 h at RT, followed by three washing steps with PBST0.05. In duplicates different volumes of culture filtrates or cell extracts diluted in PBS (exact amounts described in results) were applied to the antibody coated cavities and incubated for 1.5 h at RT, followed by three washing steps with PBST0.05. Detection of bound antigen was performed with HRP conjugated scFv-Fc (exact dilutions described in results) in PBST-B for 1.5 h at RT, followed by three washing steps with PBST0.05. The assay was further processed as described above.

3.7 Cell culture

3.7.1 Cultivation and transient transfection of HEK293-6E (scFv-Fc production)

HEK293-6E cells were cultivated in chemical defined F17 medium supplemented with 1 g L^{-1} pluronic F68, 4 mM L-glutamine and 25 mg L^{-1} G418 at 37 °C and 110 rpm with 5 % CO_2 as described in (Jäger *et al.*, 2013).

Transient production in suspension HEK293-6E cells was performed as described in (Schirrmann and Büssow, 2010). Briefly, two days prior to transfection 5 × 10^5 cells mL^{-1} HEK293-6E were seeded into 25 mL F17 medium (without G418) into 125 mL polycarbonate Erlenmeyer flasks with ventilation membrane caps and incubated at 37 °C and 110 rpm in an orbital shaker. A total of 1 µg high quality plasmid-DNA and 2.5 µg polyethyleneimine (PEI) per mL culture volume were prepared in 1/10 volume of fresh F17 medium, incubated for 15 – 30 min at RT and applied to the cells which were further cultured for 48 h. Then 0.5 % (w/v) of tryptone N1 were added as described in (Pham *et al.*, 2006) together with one additional volume fresh culture medium. Cultivation was carried on for another 3 days. Subsequently a final concentration of 1 % (v/v) of FCS was added for stabilization of scFv-Fc fusion proteins before harvesting at 3,220 x g and 4 °C for 30 min.

3.7.2 Purification of Fc-fusions with Protein A

All scFv-Fc antibodies were purified by protein A affinity purification using 1 mL Bio-Scale Mini UNOsphere SUPrA Cartridges and the semiautomated Profinia 2.0 system (Bio-Rad, Germany) according to the manufacturer's instructions.

3.7.3 Cultivation of Lx143

A-LAM IgM producing hybridoma cells were cultivated for 14 days in DMEM medium supplemented with 10 % (v/v) FCS and 1 % (v/v) Pen/Strep at 37 °C, 5 % CO_2 and 95 % humidity in a 75 cm^2 cell culture flask.

3.8 Lateral flow immuno assays

Colloidal gold-antibody conjugates (Lionex GmbH) were used as detection reagents in lateral flow immuno assays (LFIA) assembled as described below:

5 – 80 µL cm^{-1} of colloidal gold-antibody conjugates were dispensed onto 8 mm glass fibre pads using the xyz-dispenser (Biodot) and dried at 37 °C. 1 µL cm^{-1} dilution of capture antibody (sandwich assay) or antigen (direct assay) in PBS were dispensed in a line onto Unisart CN 95 nitrocellulose membranes (20 mm) that were already assembled onto 300 mm backing cards (DIMA), and dried at 37 °C. The glass fibre conjugate pads

were glued onto the backing cards, overlapping the nitrocellulose membrane at the connection point for ~ 0.5 – 1 mm. Then cellulose fibre pads (Millipore) were glued onto the backing cards as sample and wicking pad, overlapping the nitrocellulose membrane at the connection point for ~ 0.5 – 1 mm. The assembled cards were cut into strips of 0.4 cm width with the CM4000 guillotine-cutter (Biodot). The test strips were placed into Lateral Flow Strip Test (LFST) cassettes (Jieyi Biotechnology).Cassettes were packaged and sealed into LDPE bags (Viking) including Dessicant bags (Wisepac) for humidity control and stored at RT until further use.

3.8.1 Capillary flow procedure

120 µL (direct assay) or 150 µL (sandwich assay) of samples were applied onto the sample well of the LFIA cassettes and allowed to process with lateral flow for 15 – 20 min at RT before scanning or photographing.

4 Results

4.1 LAM

4.1.1 Cloning antibodies from Lx143 hybridoma cells

The isolation of the variable regions of the LAM specific IgM antibody Lx143 (variable light chain (V_L) and variable heavy chain (V_H)) was performed as described in (Toleikis *et al.*, 2004; Dübel *et al.*, 1994) with modifications. An overview of the procedure is outlined in Figure 8.

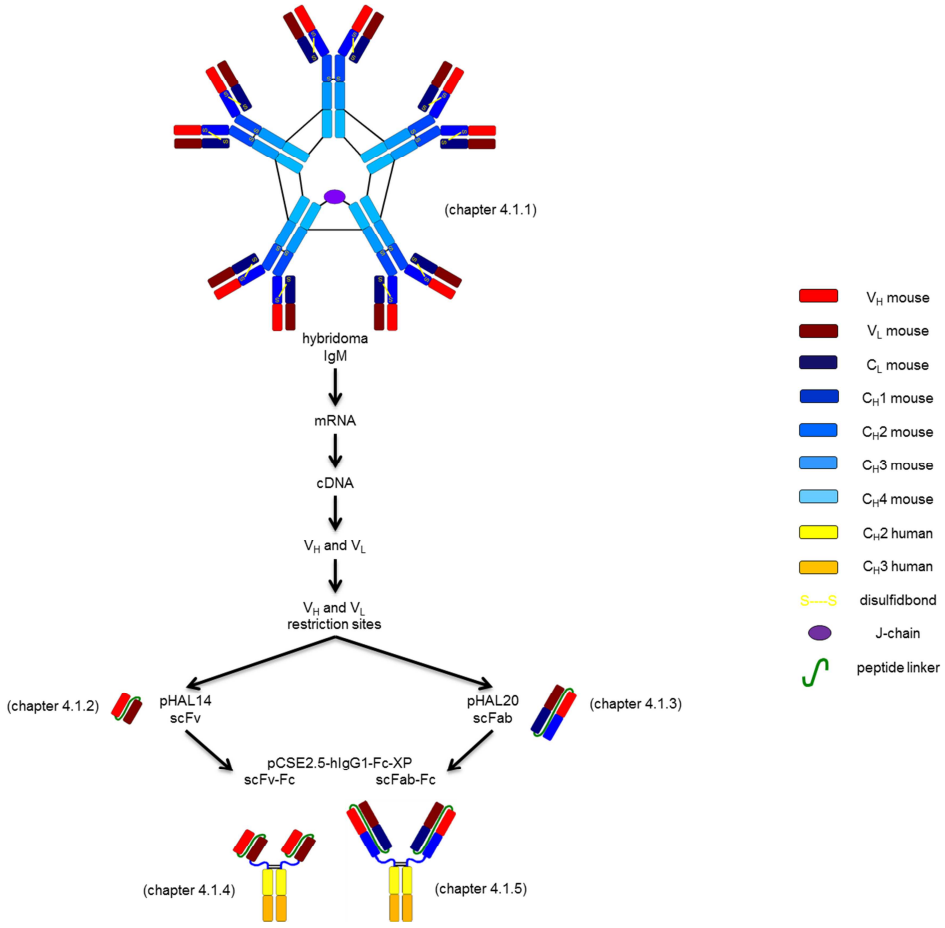


Figure 8: Overview cloning α-LAM antibodies from α-LAM hybridoma cells.
Antibody symbols adopted from M. Hust with permission.

First total RNA was isolated from Lx143 hybridoma cells. Targeting the poly A tail of mRNA cDNA was synthesized. The V_H and V_L genes were amplified from the cDNA using wobble oligonucleotides targeting different mouse gene subfamilies. Interestingly six of seven oligonucleotide combinations resulted in a PCR product of about 350 bp (estimated size of a variable region) which can be followed in Figure 9.

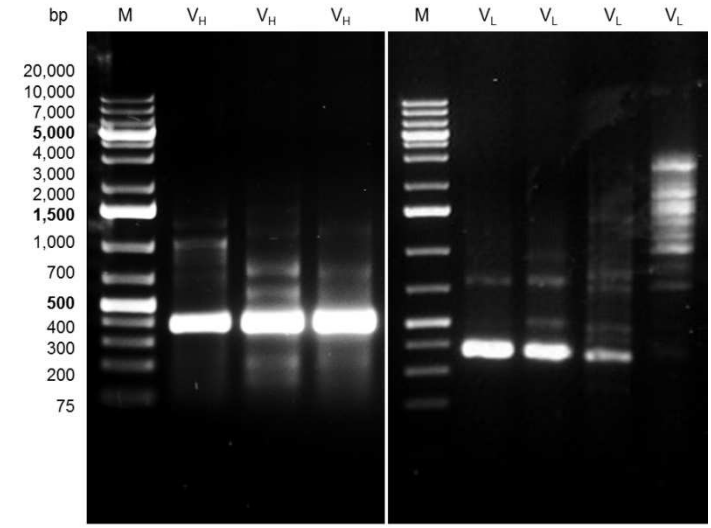


Figure 9: Amplification of variable regions of heavy and light chain from α-LAM IgM Lx143 cDNA.
5 µL of PCR product from various oligonucleotide primer combinations and 3 µL of marker (M) were separated on 1.5 % agarose gel and visualized with GelStar.

All received antibody genes were subcloned into pCR2.1-TOPO and sequenced. The analysis of the obtained results with VBASE2 and MultAlign showed that all isolated heavy/light chains were originated from the same subfamily, gene and allel. It was reasoned that there was only one functional heavy and light chain combination.

N-terminal sequencing of the α-LAM IgM was performed at the Helmholtz Centre for Infection Research, Germany. The received data showed an antibody sequence not identical with any of the sequences extracted from the hybridoma cells. There were minimum six N-terminal amino acids (three in V_H, three in V_L) exchanged. To obtain the correct amino acid order, the extracted sequences were altered by PCR. The α-LAM V_H and V_L genes were then provided with appropriate restriction sites and subcloned into pHAL14 to complete the linker and form a functional scFv.

4.1.2 A-LAM scFv

E.coli XL1Blue MRF' was transformed with the α-LAM scFv in pHAL14. Positive clones were screened by cultivation on selective medium and colony PCR. Confirmation of the constructed scFv sequence was carried out by DNA-sequencing. Soluble scFv were produced in MTP and analysed by screening ELISA and SDS-PAGE followed by α-c-Myc-tag immunoblot. The ELISA was performed with controls of both antigen (α-LAM IgM detection) and the scFv production/detection system (coproduction of α-lysozyme scFv D1.3, BSA negative control). No signal could be obtained by α-LAM scFv in ELISA (data not shown), but a protein band of ~ 32 kDa could be detected in the culture supernatant after production in MTP via immunostaining (Figure 10).

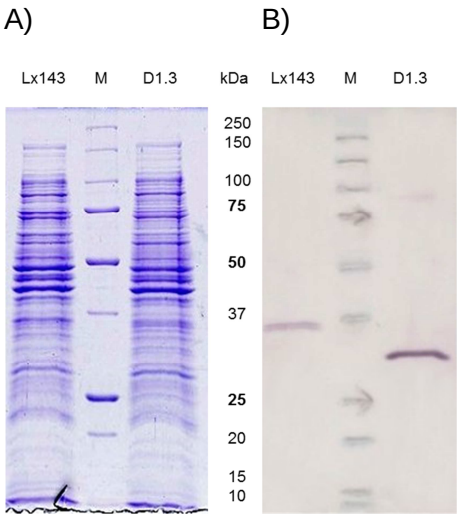


Figure 10: A) Coomassie staining and B) α-c-Myc-tag immunoblot of culture supernatants from production of soluble α-LAM scFv in MTP.
18 µL culture supernatant and 3 µL marker (M) were separated on reducing 12 % SDS-PAGE and rather stained with coomassie or electro blotted to a PVDF membrane. ScFv on the blot were detected using mouse α-c-Myc-tag IgG(9E10) followed by goat α-mouse IgG(Fc)-HRP, development with TMB. D1.3 represents the positive control.

For further characterization of the scFv a production in 1 L scale (shaking flasks) was carried out. There was no scFv detectable in the eluat fractions after Ni-NTA by SDS-PAGE and silver staining (data not shown). The expression of the scFv was checked by means of SDS-PAGE followed by α-c-Myc-tag immunoblot. A slight protein band of scFv could be detected in the culture supernatant and in the PPP and OSP, whereas a strong band was visible in the pellet after PPP and OSP production (data not shown). This surveillance indicates instability and inclusion body formation of the scFv in *E. coli*.

Hence a change of antibody format from scFv to scFab seemed necessary, to stabilize the antibody. For that purpose the α-LAM V_H and V_L were provided with appropriate restriction sites and subcloned to pHAL20 to complete the linker and to equip the variable regions with mouse C_H1 and C_L for the formation of a functional scFab.

4.1.3 A-LAM scFab

Soluble α-LAM scFab were produced in MTP. Culture supernatant and PPP + OSP were prepared and tested in the same ELISA setting as described for the α-LAM scFv. No reaction of the soluble scFab with the antigen could be detected (data not shown). The production of scFab was analysed by SDS-PAGE followed by immunoblot and could be verified (Figure 11).

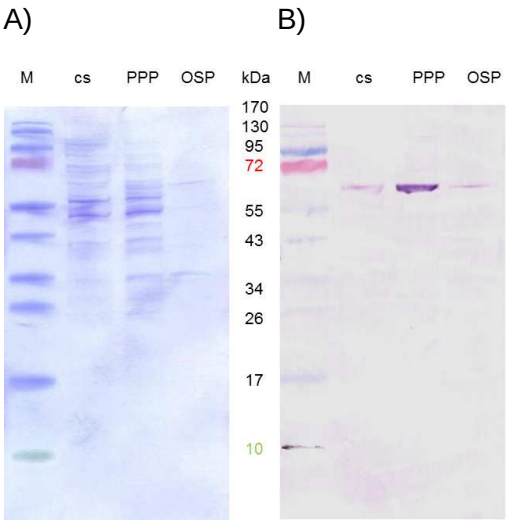


Figure 11: A) Coomassie staining and B) α-c-Myc-tag immunoblot of culture supernatant, PPP and OSP from production of soluble α-LAM scFab in MTP.
18 µL culture supernatant (cs), PPP or OSP and 6 µL marker (M) were separated on reducing 12 % SDS-PAGE and rather stained with coomassie or electro blotted to a PVDF membrane. Immunostain was performed as described in Figure 10.

For further characterization of the scFab a production in 1 L scale was carried out. There was no scFab detectable in the eluat fractions after Ni-NTA by SDS-PAGE and silver staining (data not shown). Comparable to the scFv only small amounts of protein were found in the culture supernatant, PPP and OSP analysed by immunoblot. Whereas a great portion of scFab was found in the pellet after PPP and OSP preparation (data not shown). It was concluded that the scFab could not be purified. On that account the fusion of the single chain α-LAM antibodies with a human IgG1 Fc part was focused on to first stabilize the construct and second to fetch an avidity effect and by that means an increase of affinity.

The fusion of α-LAM scFv with a human IgG1 Fc part was performed by subcloning the scFv into the eukaryotic expression vector pCSE2.5-hIgG1-Fc-XP.

4.1.4 A-LAM scFv-Fc

HEK293-6E cells were transient transfected with the α-LAM scFv-Fc in pCSE2.5-hIgG1-Fc-XP. No scFv-Fc could be purified (triplicate approach, with technical assistance of Franziska Resch (TU-BS)). To receive further stability the format change from scFv-Fc to scFab-Fc was carried out by subcloning the scFab into pCSE2.5-hIgG1-Fc-XP.

4.1.5 A-LAM scFab-Fc

HEK293-6E cells were transient transfected with the α-LAM scFab-Fc in pCSE2.5-hIgG1-Fc-XP. A-LAM scFab-Fc was purified from the culture supernatant via Protein A (with technical assistance of Franziska Resch (TU-BS)). A protein yield of 24 mg L^{-1} was

determined by a Lowry protein assay. The obtained antibody solution was analysed by SDS-PAGE followed by silver staining, α-human IgG(Fc) immunoblot and reducing gel analysis via Tape Station (Figure 12). No degradation could be detected in the antibody preparation by either means. Tape station analysis issued a purity of 96.8 % for the target 74 kDa band.

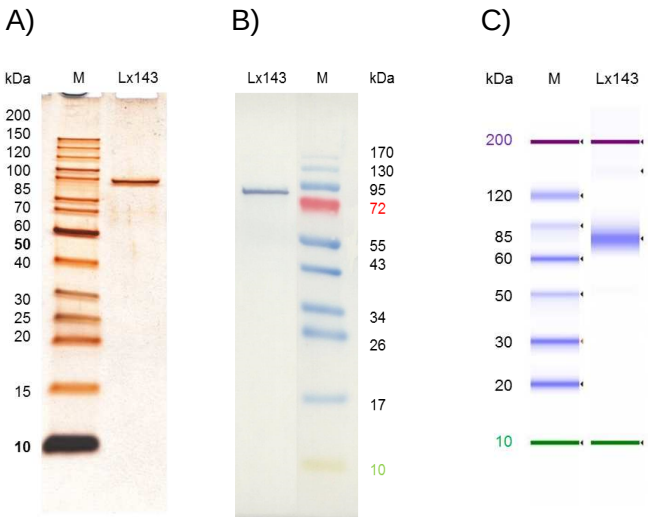


Figure 12: A) Silver staining, B) α-human IgG(Fc) immunoblot and C) Tape Station analysis of purified α-LAM scFab-Fc.
A) 75 ng of purified Lx143 α-LAM scFab-Fc and 1 µL of marker (M) were separated on reducing 12 % SDS-PAGE and silver stained. B) 200 ng of purified Lx143 α-LAM scFab-Fc and 8 µL of marker were separated on reducing 12 % SDS-PAGE and electro blotted to a PVDF membrane. After blocking, human IgG(Fc) was detected using goat α-human IgG(Fc)-HRP, development with TMB, C) scFab-Fc were directly analysed by reducing gel analysis via Tape Station (with technical assistance of Wiebke Prilop (Lionex GmbH)).

The antigen recognition of the α-LAM scFab-Fc was analysed by titration ELISA (Figure 13). The antibody bound BCG derived LAM specifically.

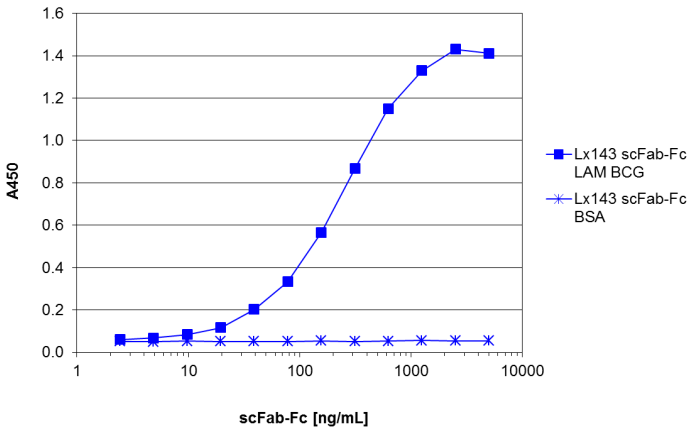


Figure 13: Titration ELISA of α-LAM scFab-Fc.
A dilution series of scFab-Fc was used for detection of directly coated antigen (LAM BCG) or BSA (control), detection of bound scFab-Fc with goat α-human IgG(Fc)-HRP, development with TMB.

Further, the reaction with Mtb and BCG cell extracts and culture filtrates was analysed by indirect ELISA (Figure 14). In this assay, the α-LAM scFab-Fc bound Mtb and BCG derived LAM, whereupon the reaction with Mtb LAM was slightly more intense. In addition, LAM was detected in Mtb and BCG cell extracts and Mtb culture filtrate.

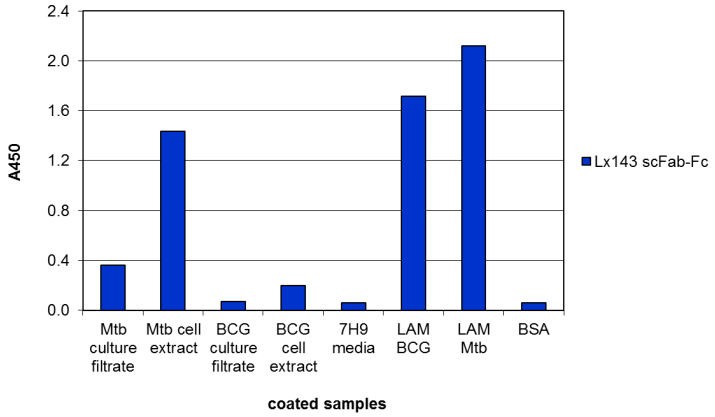


Figure 14: Reaction of α-LAM scFab-Fc with Mtb/BCG cell extracts and culture filtrates determined by indirect ELISA.
100 ng/100 µL of the different samples were directly coated to the well, reaction was determined with a scFab-Fc concentration of 2.5 µg mL^{-1}, detection of bound scFab-Fc with goat α-human IgG(Fc)-HRP, development with TMB.

4.2 Selection of antibodies from HAL7/8

ScFv were isolated *in vitro* from the human naïve libraries HAL7/8 by panning. Soluble monoclonal antibodies were produced in MTP and specific antigen binding was analysed by screening ELISA. Individual binders were identified by DNA-sequencing.

No binders were received concerning LAM or ESAT-6 (Table 29). Six individual AlaDH binders were isolated, but the specific antigen recognition could not be reproduced in following assays (data not shown).

Table 29: Overview selection of antibodies from HAL7/8.

antigen	independant pannings*	screened clones	hits screening ELISA	clones sequenced	individual sequences
LAM	3	276	0	0	0
ESAT-6	4	418	0	0	0
AlaDH	2	184*	9	6	6
16 kDa	4†	644*	135	21	7
CFP-10	3	322	7	7	3
85 A	3	322	16	11	4
85 B	2	184	51	11	5
85 D	2	184	15	10	3
total	19	2534	233	66	28

*with technical assistance of Saskia Helmsing (TU-BS) and Dr. Wulf Oehlmann (Lionex GmbH), †HAL4/7/8

4.2.1 16 kDa

4.2.1.1 A-16 kDa scFv

Seven individual 16 kDa binders were identified by panning of HAL4/7/8 and subsequent screening ELISA followed by DNA sequencing (data not shown). All these antibodies have the same variable gene segment of the heavy chain (HV). In addition to that, respectively six out of seven binders have the same joining gene segment of the light chain (LJ), the same variable gene segment of the light chain (LV) or the same joining gene segment of the heavy chain (HJ) (Table 30).

Table 30: Comparison of heavy and light chain gene segments of α-16 kDa antibodies.

	heavy chain			light chain	
clone	**HV**	**D**	**HJ**	**LV**	**LJ**
SH365-E4	IGHV3-15*01	IGHD3-10*01	IGHJ4*02	IGLV1-47*01	IGLJ3*01
SH365-H4	IGHV3-15*01	IGHD5-24*01	IGHJ4*02	IGLV1-47*01	IGLJ3*01
SH365-C8	IGHV3-15*01	IGHD6-6*01inv	IGHJ4*02	IGLV1-47*01	IGLJ3*01
L16-3-D12	IGHV3-15*01	IGHD2-8*01	IGHJ4*02	IGLV1-47*02	IGLJ3*01
L16-3-E12	IGHV3-15*01	IGHD2-2*02inv	IGHJ4*02	IGLV1-47*01	IGLJ3*02
SH451-C11	IGHV3-15*01	IGHD6-6*01inv	IGHJ4*02	IGLV1-47*01	IGLJ3*01
SH451-F11	IGHV3-15*01	IGHD5-24*01	IGHJ5*02	IGLV1-47*01	IGLJ3*01

Abbreviations: HV: variable (V) gene segment of the heavy chain; D: diversity gene segment; HJ: joining (J) gene segment of the heavy chain; LV: variable gene segment of the light chain; LJ: joining gene segment of the light chain

All scFv were subcloned to the prokaryotic expression vector pOPE101-XP, produced in 1 L scale (shaking flasks), PPP and OSP were prepared and purified over Ni-NTA IMAC. ScFv containing fractions of the eluate (determined by SDS-PAGE followed by coomassie staining, data not shown) were pooled and rebuffered into PBS using preparative SEC (data not shown). The protein concentration of the received scFv preparations was determined by a Lowry assay and the yield per litre was calculated (Table 31). The purity of the antibody solutions was analysed by reducing gel analysis via Tape Station and SDS-PAGE followed by silver staining. There were no other protein bands than the target scFv band (~ 30 kDa) visible in the silver stained SDS-PAGE (Figure 15: A). Tape Station analysis revealed a different protein pattern with up to three additional bands (Figure 15: B), indicating the presence of contamination (in form of *E. coli* proteins), scFv aggregates or degradation products of these antibodies.

Table 31: Overview purification of α-16 kDa scFv.

scFv	yield [mg L^{-1}] [a]	purity [%] [b]	monomer [%] [c]
SH365-E4	33.50	95.2	93.97
SH365-H4	5.34	100.0	88.10
SH365-C8	46.04	100.0	92.94
L16-3-D12	36.30	80.4	93.72
L16-3-E12	16.11	100.0	97.40
SH451-C11	2.32	88.9	74.31
SH451-F11	1.98	92.3	83.89

a) determined by Lowry protein quantification assay; b) determined by reducing Tape Station analysis, percent of integrated area; c) determined by analytical SEC, percent of integrated area

A) B)

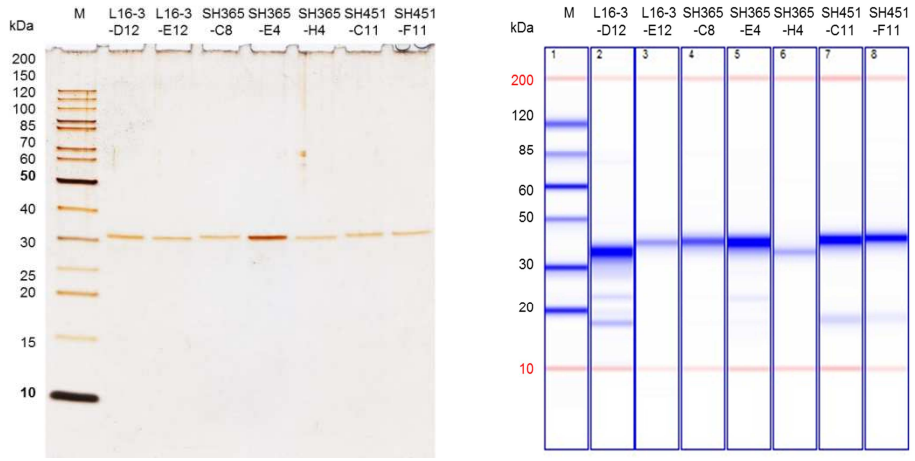


Figure 15: A) Silver staining and B) Tape Station analysis of purified α-16 kDa scFv.
A) 100 ng of purified scFv and 1 µL of marker were separated on reducing 12 % SDS-PAGE and silver stained. B) scFv were directly analysed by reducing gel analysis via Tape Station (with technical assistance of Wiebke Prilop (Lionex GmbH)).

A disadvantage of the scFv format is the possible formation of associates promoted by high antibody concentrations (Wörn and Plückthun, 2001). Dimers and multimers may contain more than one paratope and, due to the avidity affect, exhibit stronger apparent binding characteristics than the corresponding monomers. Aggregates might handicap the antigen binding due to sterical inhibition or on account of a wrong conformation of the antigen binding site. Therefore the quaternary structures of the scFv in PBS pH 7.4 were analysed via analytical SEC. All antibodies showed a dominant peak at an estimated size of ~30 kDa which reflects the theoretical size of a monomeric scFv. In addition to that, all scFv preparations displayed another peak (to an extent of 26 % of the integrated area, compare Table 31) with an estimated size ranging from 65 – 90 kDa that could indicate the presence of di- and trimers. Exemplary the analytical SEC of L16-3-E12 is displayed in Figure 16.

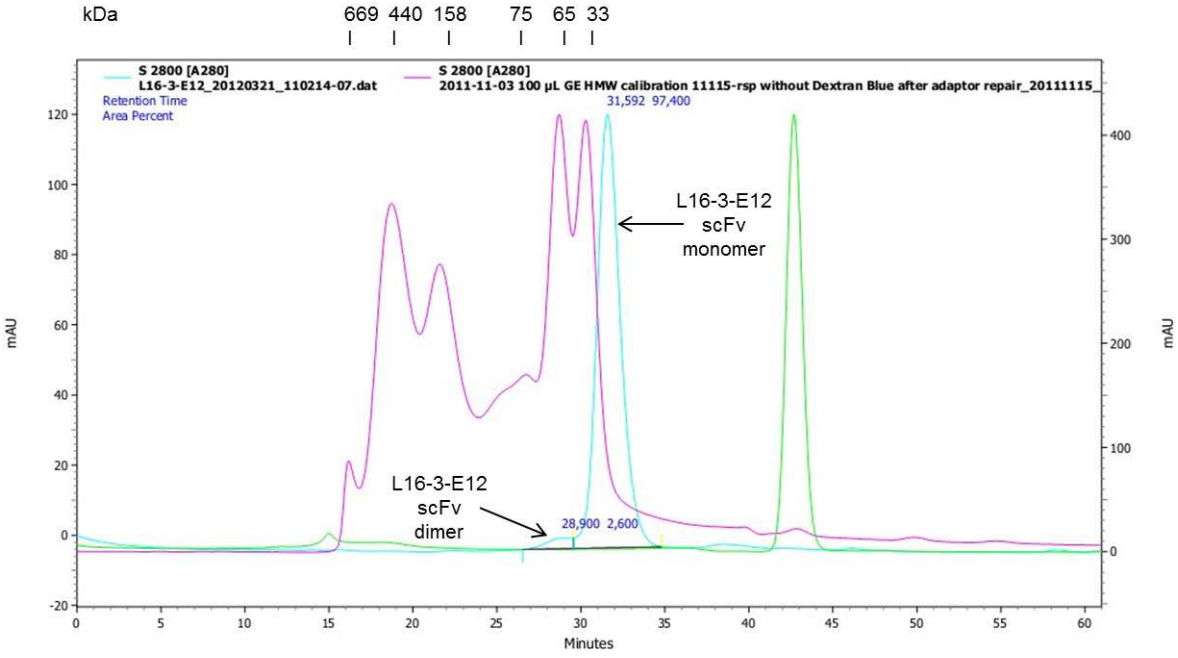


Figure 16: Analytical SEC of L16-3-E12 scFv.
100 µL of L16-3-E12 scFv (1.24 mg mL^{-1}) were applied to Superdex 200 10/300 GL column, running buffer was PBS pH 7.4. Standards: 1. 669 kDa 19 min, 2. 440 kDa 22 min, 3. 158 kDa 27 min, 4. 75 kDa 29 min, 5. 65 kDa 30 min, 6. 33 kDa 33 min; pink line: molecular standards, blue line: sample, green line: conductivity. Analysis was performed with technical assistance of Dr. Ralf Spallek (Lionex GmbH).

The antigen binding of the scFv was analysed by titration ELISA (Figure 17). All antibodies bound 16 kDa specifically in this assay.

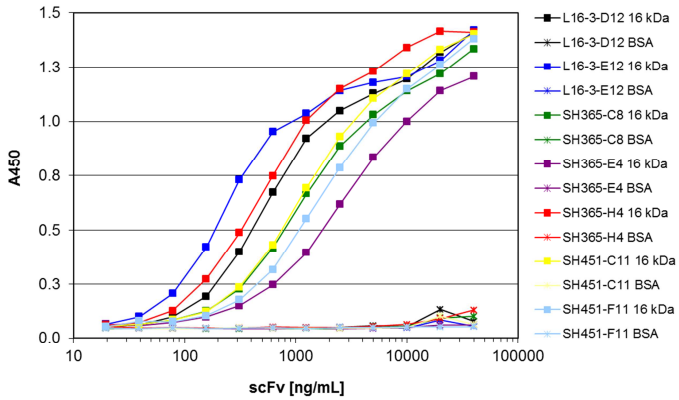


Figure 17: Titration ELISA of α-16 kDa scFv.
Different dilutions of scFv were used for detection of 100 ng directly coated antigen (16 kDa) or BSA (negative control), detection of bound scFv with mouse α-c-Myc-tag 9E10 IgG followed by goat α-mouse IgG(Fc)-HRP, development with TMB.

Cross reactivity with other Mtb antigens was determined by indirect ELISA (Figure 18). L16-3-E12 and L16-3-D12 showed a slight cross reactivity with 85 complex antigens, but delivered the highest signals with the 16 kDa antigen at the same time.

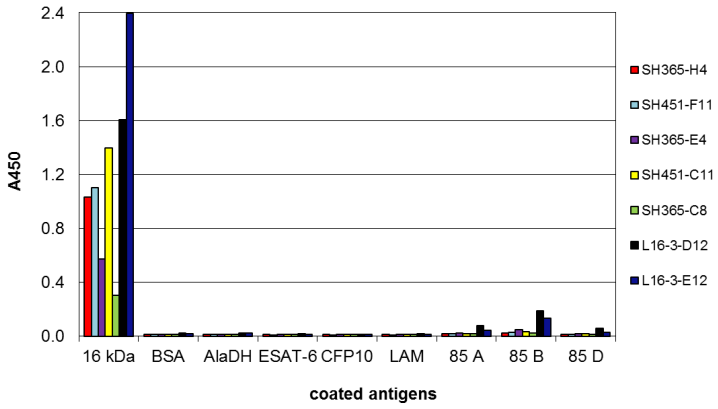


Figure 18: Cross reactions of α-16 kDa scFv with other Mtb antigens determined by ELISA.
100 ng of the diffferent antigens in PBS were directly coated to the well, cross reaction was determined with a scFv concentration of 1.25 µg mL^{-1}, detection of bound scFv with mouse α-c-Myc-tag 9E10 IgG followed by goat α-mouse IgG(Fc)-HRP, development with TMB.

To determine whether the α-16 kDa antibodies recognized continuous or conformational epitopes, α-16 kDa immunoblots were performed (data not shown). In these assays all antibodies bound non-reduced (10 min 56 °C, no DTT) antigen strongly and reduced (10 min 96 °C, + 1 M DTT) antigen weakly, indicating the recognition of at least partial continuous epitopes.

Epitope mapping of the α-16 kDa scFv was carried out on PepSpot membranes (Figure 19). The reaction of the antibodies with the PepSpots was weak. In addition a strong cross reaction of the secondary antibody with the spots 1, 2, 6, 7, 8, 23, 24 and 29 was monitored (Figure 19: red encircled area), which could not be avoided by changing the antibody or the visualization system. Nevertheless it could be observed that all scFv reacted with the same three spots on the membrane (Figure 19: spots 20, 21 and 22) and hence it was reasoned that they recognize the same continuous sequence. Through the overlap of the peptide sequences (Table 32) the epitope was determined to "KDFDGRS", which is located from amino acid 85 – 91 in the 16 kDa antigen sequence.

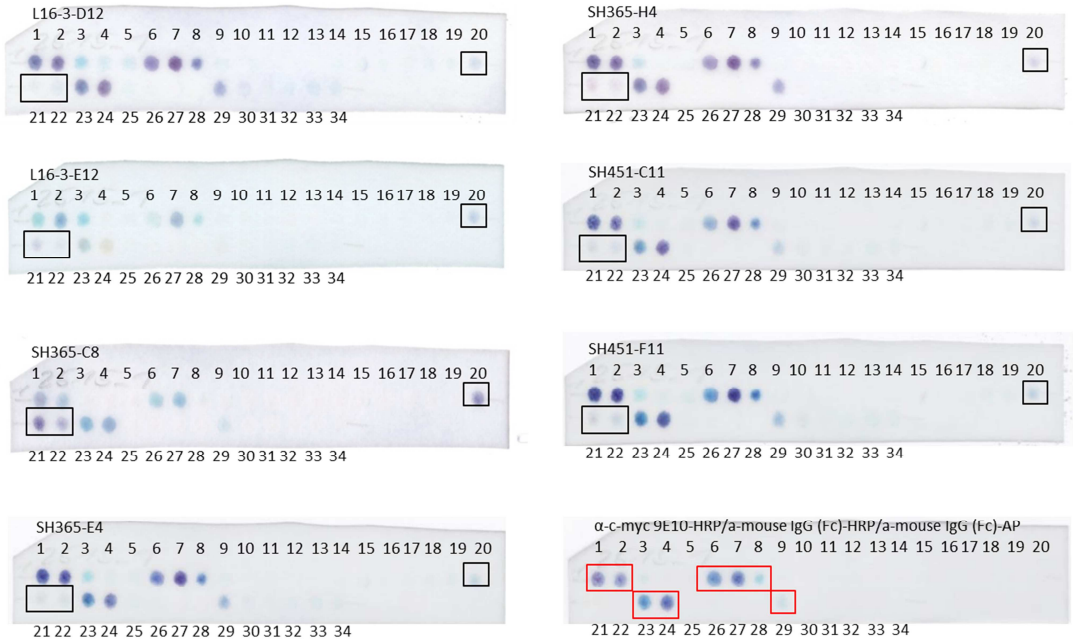


Figure 19: Epitope mapping of α-16 kDa scFv with PepSpot membrane.
Membrane was incubated with respectively 0.67 µg mL^{-1} antibody, detection of bound scFv with mouse α-c-Myc-tag 9E10 IgG followed by goat α-mouse IgG(Fc)-HRP, development with TMB. Immunostained spots are encircled.

Table 32: Epitope mapping of α-16kDa scFv, amino acid sequences of peptides on 16 kDa PepSpot membrane.
The immunostained spots and the identified epitope are highlighted.

spot no.	peptide
19	GQLTIKAERTEQKDF
20	IKAERTEQKDFDGRS
21	RTEQKDFDGRSEFAY
22	KDFDGRSEFAYGSFV
23	GRSEFAYGSFVRTVS

To create a ranking of the α-16 kDa scFv, their binding characteristics for the antigen were determined by surface plasmon resonance (SPR). The antibody with the lowest K_D and therefore the highest affinity was determined to be L16-3-E12 (Table 33).

Table 33: Affinity measurement of α-16 kDa scFv via SPR.
List of binding parameters for the reviewed antibodies. The dissociation constant K_D is the common dimension for the description of affinity.

scFv	ka [M^{-1} s^{-1}]	kd [s^{-1}]	R_{max} [RU]	K_A [M^{-1}]	K_D [M]	X^2	% X^2 (R_{max})*
L16-3-D12	1.38E+03	3.82E-04	20.6	3.62E+06	2.76E-07	0.050	0.24
SH365-E4	2.35E+04	4.90E-03	10.2	4.80E+06	2.08E-07	0.453	4.44
SH451-C11	1.32E+04	1.68E-03	16.0	7.87E+06	1.27E-07	0.184	1.15
SH365-H4	2.99E+04	3.37E-03	16.9	8.88E+06	1.13E-07	0.201	1.19
SH451-F11	1.58E+04	1.78E-03	15.8	8.88E+06	1.13E-07	0.193	1.22
SH365-C8	2.66E+04	1.98E-03	14.7	1.34E+07	7.44E-08	0.062	0.42
L16-3-E12	1.48E+04	1.57E-04	13.2	9.44E+07	1.06E-08	0.140	1.06

*X^2 should be less than 5 % of R_{max}

Because all α-16 kDa scFv presumably recognized the same epitope only the antibody with the highest affinity for the antigen was chosen for further biochemical characterization. L16-3-E12 was subcloned to pCSE2.5-hIgG1-Fc-XP to equip it with a human IgG01 Fc part for better stability, detection possibilities and conjugation procedures.

4.2.1.2 A-16 kDa scFv-Fc

HEK293-6E cells were transient transfected with the L16-3-E12 scFv-Fc in pCSE2.5-hIgG1-Fc-XP. The scFv-Fc was purified from the culture supernatant via Protein A (with technical assistance of Franziska Resch (TU-BS)). A yield of 112 mg L^{-1} was determined by a Lowry protein assay. The obtained antibody solution was analysed by SDS-PAGE followed by silver staining, α-human IgG(Fc) immunoblot and reducing gel analysis via Tape Station (Figure 20). No degradation could be detected in the antibody preparation by either means. Tape station analysis revealed two faint additional protein bands at 199 and 238 kDa, issuing a purity of 96.6 % for the 59 kDa scFv-Fc band.

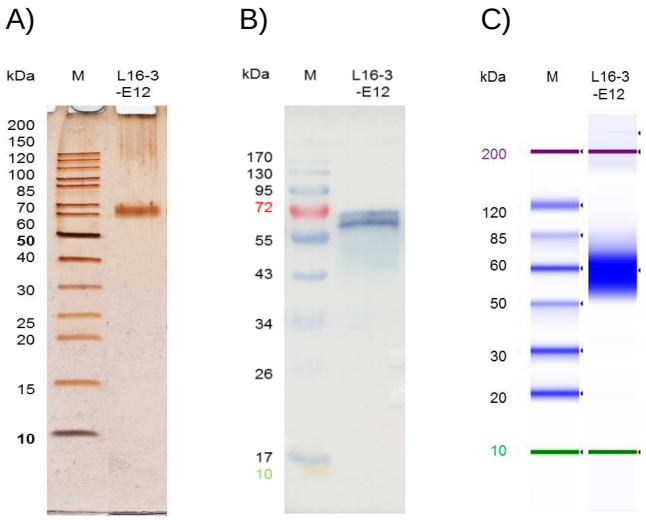


Figure 20: A) Silver staining, B) α-human IgG(Fc) immunoblot and C) Tape Station analysis of purified L16-3-E12 scFv-Fc.
Analysis carried out as described in Figure 12.

The antigen recognition of L16-3-E12 scFv-Fc was analysed by titration ELISA (Figure 21). In this assay, L16-3-E12 bound the antigen specifically.

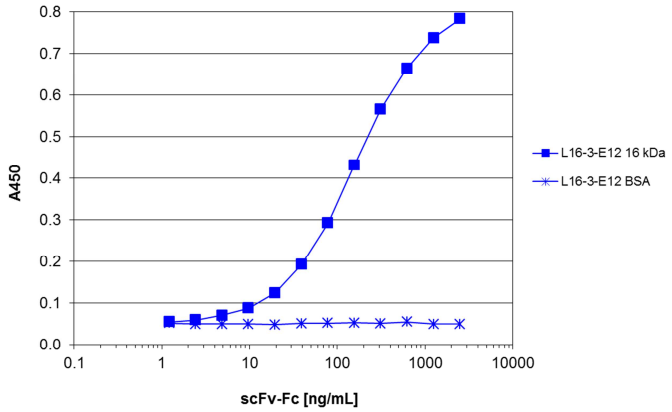


Figure 21: Titration ELISA of α-16 kDa L16-3-E12 scFv-Fc.
A dilution series of scFv-Fc was used for detection of directly coated antigen (16 kDa) or BSA (negative control), detection of bound scFv-Fc with goat α-human IgG(Fc)-HRP, development with TMB.

The antigen detection limit of L16-3-E12 scFv-Fc, meaning the lowest antigen concentration triggering a signal in ELISA distinguishable from the negative control, was determined to ~ 10 ng mL^{-1} by antigen titration ELISA (Figure 22).

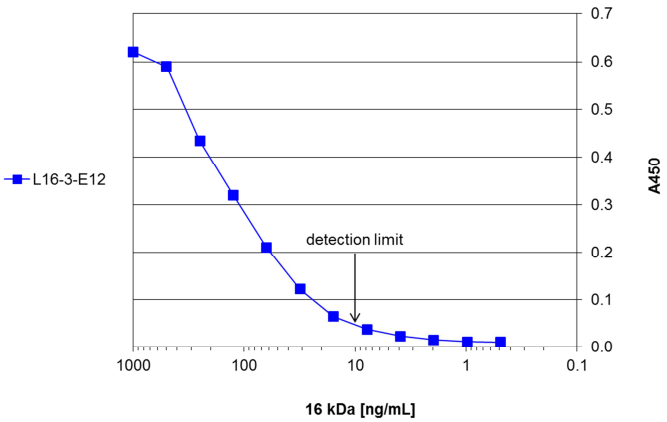


Figure 22: Antigen titration ELISA with α-16 kDa L16-3-E12 scFv-Fc.
Different dilutions of antigen (16 kDa) were directly coated to the wells. Antigen detection with L16-3-E12 scFv-Fc at a concentration of 150 ng mL^{-1}, detection of bound scFv-Fc with goat α-human IgG(Fc)-HRP, development with TMB. Negative control BSA A_{450} = 0.01.

Recognition of Mtb and BCG cell extracts and culture filtrates was analysed by indirect ELISA (Figure 23). A slight reaction of L16-3-E12 scFv-Fc with Mtb culture filtrate and cell extract was discovered.

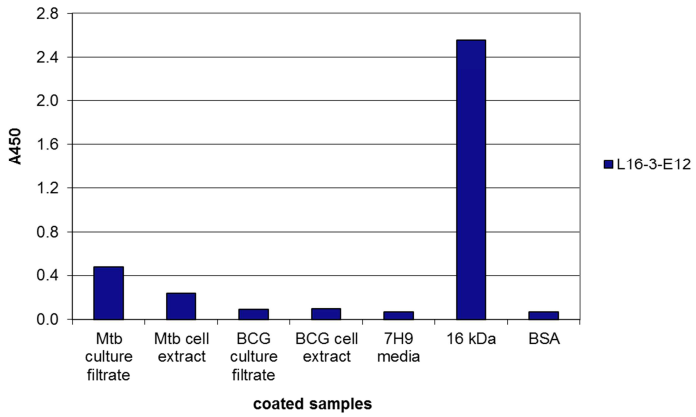


Figure 23: Reaction of α-16 kDa L16-3-E12 scFv-Fc with Mtb/BCG cell extracts and culture filtrates determined by indirect ELISA.
100 ng/100 µL of the different samples were directly coated to the well, reaction was determined with a scFv-Fc concentration of 2.5 µg mL^{-1}, detection of bound scFv-Fc with goat α-human IgG(Fc)-HRP, development with TMB.

4.2.1.3 A-16 kDa scFv-Fc-HRP

L16-3-E12 scFv-Fc was conjugated to HRP. The necessary dilution of the HRP-conjugate was determined by direct ELISA to ~ 100 ng mL^{-1} (data not shown). The cross reactivity of the achieved antibody-HRP conjugate was analysed by immunoblot (Figure 24). The reaction with the 16 kDa antigen was slight, comparable to the reaction during

epitope mapping (compare Figure 19). Again a weak cross reaction with the Mtb antigens 85 A and 85 B, as observed before with the scFv in indirect ELISA (compare Figure 18), was detected. In exchange there was no cross reaction with human samples (sputum and serum) or *E. coli* and HEK cells.

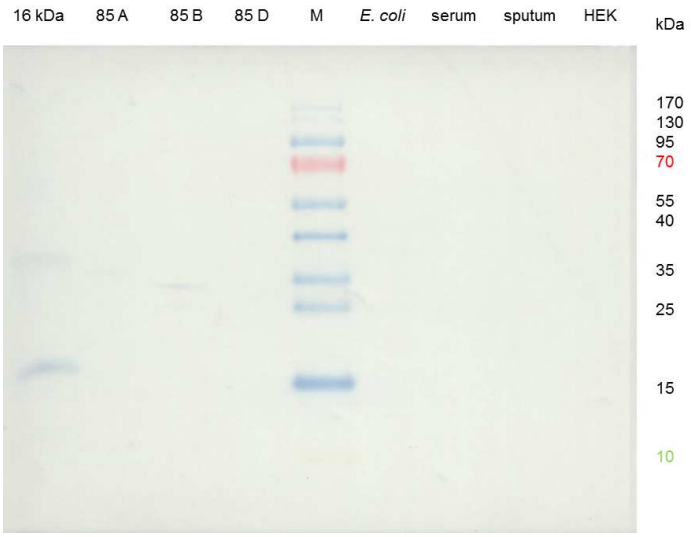


Figure 24: Cross reactions of α-16 kDa L16-3-E12 scFv-Fc-HRP in immunoblot.
500 ng of purified 16 kDa (04-3/1), 85 A (07-1/1), 85 D (07-1/3), 1 µg of purified 85 B (10-1/1), 9 µL *E. coli* BL21 (DE3) culture (OD_{600} ~7), 0.45 µL of human serum (DRK 895), 3.4 µL of human sputum (B-Sp-0029), $4.5x10^4$ cells HEK293-6E, 5 µg *M. tuberculosis* H37Rv extract and 8 µL of marker were separated on reducing 12 % SDS-PAGE and electro blotted to a PVDF membrane. After blocking, membrane was incubated with L16-3-E12 scFv-Fc-HRP at a concentration of ~ 0.6 µg mL^{-1}, development with TMB.

Nanoelectrospray mass spectrometry revealed the dodecameric structure of the 16 kDa antigen of *M. tuberculosis* recombinantly expressed in and purified from *E. coli* (Kennaway, 2005). Analytical SEC of the available recombinant 16 kDa from Lionex GmbH offered a partial multimeric conformation (Figure 25). Therefore, even if there was only one antibody recognizing one epitope on the 16 kDa antigen, a sandwich ELISA was performed. Sampling different 16 kDa batches (with a multimere peak in SEC) no signal could be obtained in α-16 kDa sandwich ELISA (data not shown). Furthermore *M. tuberculosis* culture filtrate (cultured three months in 7H9 + ADC + Tween at 37 °C) was tested in α-16 kDa sandwich ELISA to no avail (data not shown).

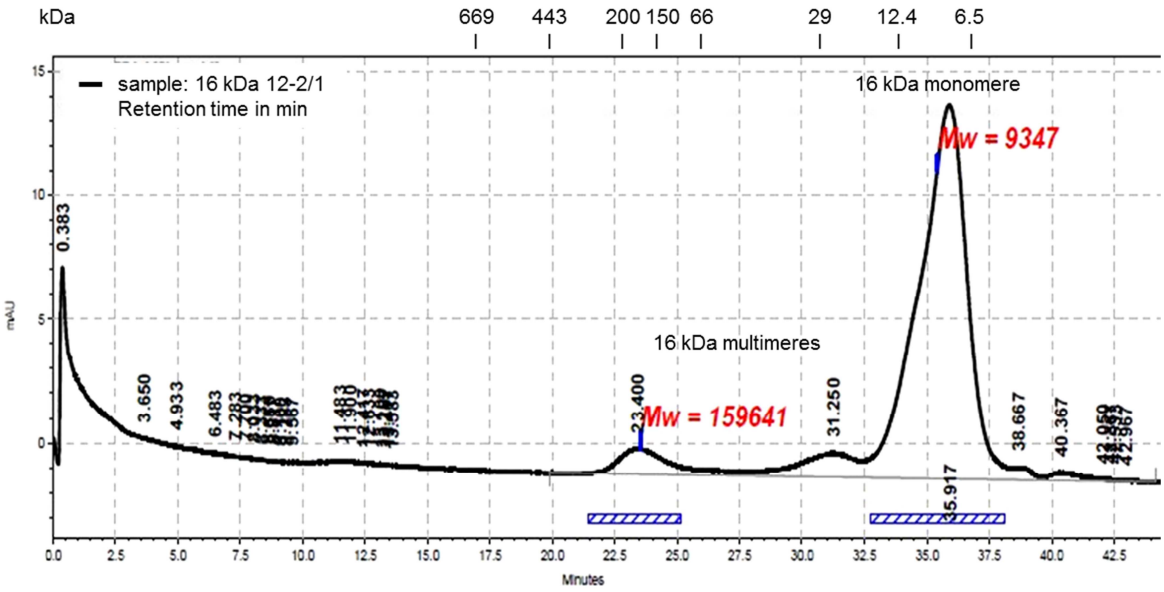


Figure 25: Analytical SEC of 16 kDa batch 12-2/1.
500 µL of 16 kDa (1 mg mL^{-1}) were applied to Superdex 200 10/300 GL column, running buffer was PBS pH 7.4. Standards: 1. 669 kDa 17 min, 2. 443 kDa 20 min, 3. 200 kDa 23 min, 4. 150 kDa 24 min, 5. 66 kDa 26 min, 6. 29 kDa 31 min, 7. 12,4 kDa 34 min, 8. 6,5 kDa 37 min. (Analysis performed with technical assistance of Wiebke Prilop (Lionex GmbH).)

4.2.2 CFP-10

4.2.2.1 A-CFP-10 scFv

Three individual CFP-10 binders were identified by panning HAL7/8 and subsequent screening ELISA followed by DNA sequencing (data not shown). The gene segments appearing in this α-CFP-10 antibodies are outlined in Table 34.

Table 34: Comparison of heavy and light chain gene segments of α-CFP-10 antibodies.

	heavy chain			light chain	
clone	**HV**	**D**	**HJ**	**LV**	**LJ**
MFU48-C8	IGHV6	IGHD3-22*01	IGHJ3*02	IGLV3-21*03	IGLJ3*02
MFU48-E1	IGHV3-23*01	IGHD3-10*01	IGHJ1*01	IGLV1-44*01	IGLJ3*01
MFU48-G4	IGHV6	IGHD3-22*01	IGHJ3*02	IGLV3-21*02	IGLJ7*01

For abbrevations see Table 30.

All scFv were subcloned to the eukaryotic transient expression vector pCSE2.5-hIgG1-Fc-XP.

4.2.2.2 A-CFP-10 scFv-Fc

HEK293-6E cells were transient transfected with the α-CFP-10 scFv-Fc in pCSE2.5-hIgG1-Fc-XP. The scFv-Fc could be successfully expressed and purified from the culture supernatant via Protein A (with technical assistance of Franziska Resch (TU-BS)). The protein yields were determined by a Lowry protein assay (Table 35). The protein band patterns of the antibody preparations could be visualized by silver stained SDS-PAGE (Figure 26: A) and reducing gel analysis via Tape Station (Figure 26: C). Additional bands at ~36 kDa and ~230 kDa were detected in all three antibody preparations. No degradation of the Fc-part could be detected in the antibody solutions by α-human IgG(Fc) immunoblot (Figure 26: B). Tape Station analysis issued a purity (scFv-Fc band) between 80 and 93 % for the explored samples (Table 35).

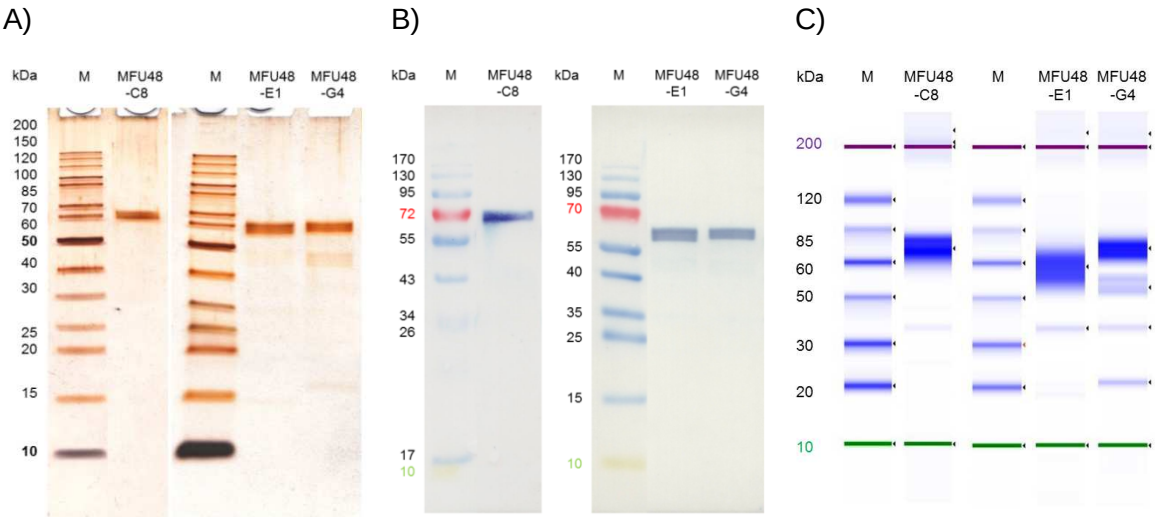


Figure 26: A) Silver staining, B) α-human IgG(Fc) immunoblot and C) Tape Station analysis of purified α-CFP-10 scFv-Fc.
Analysis carried out as described in Figure 12. Results of two experiments joined.

Table 35: Protein yield and purity of α-CFP-10 scFv-Fc preparations.

scFv-Fc	yield [mg L^{-1}][a]	purity [%][b]
MFU48-C8	111.2	89.5
MFU48-E1	157.6	93.0
MFU48-G4	173.6	79.0

a) determined by Lowry protein quantification assay; b) determined by reducing Tape Station analysis, percent of integrated area

The antigen binding was analysed by titration ELISA (Figure 27). All α-CFP-10 antibodies bound specifically.

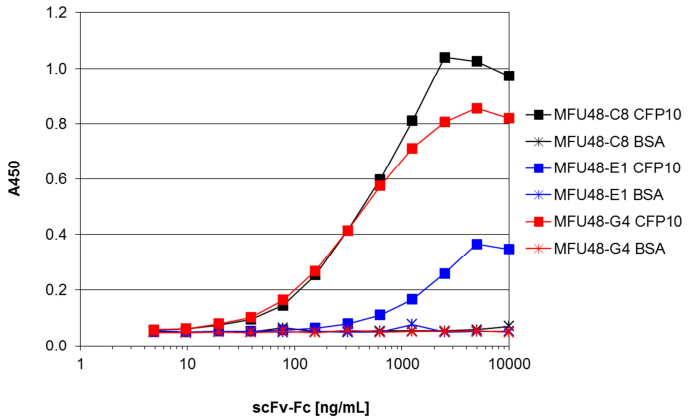


Figure 27: Titration ELISA of α-CFP-10 scFv-Fc.
Dilution series of scFv-Fc were used for detection of directly coated antigen (CFP-10) or BSA (control), detection of bound scFv-Fc with goat α-human IgG (Fc)-HRP, development with TMB.

Western blotting of CFP-10 was not feasible. Therefore it was not possible to determine whether sequential of conformational epitopes were recognized. Nevertheless, epitope

mapping of the α-CFP-10 scFv-Fc was carried out on PepSpot membranes (Figure 28). MFU48-C8 and MFU48-G4 reacted with the peptides. It was concluded, that these antibodies recognized continuous epitopes. Further, it was observed that MFU48-C8 and MFU48-G4 reacted with the same three spots on the membrane (Figure 28: spots 5, 6 and 7) and thereby recognized the same epitope. Through the overlap of the peptide sequences (Table 36) the epitope was determined to “ERISGDLKT”, which is located from amino acid 19 – 27 in the CFP-10 antigen sequence. The epitope of MFU48-E1 was not distinguishable due to the wide variety of reactions with the PepSpots, which indicated unspecific reactions or a conformational epitope.

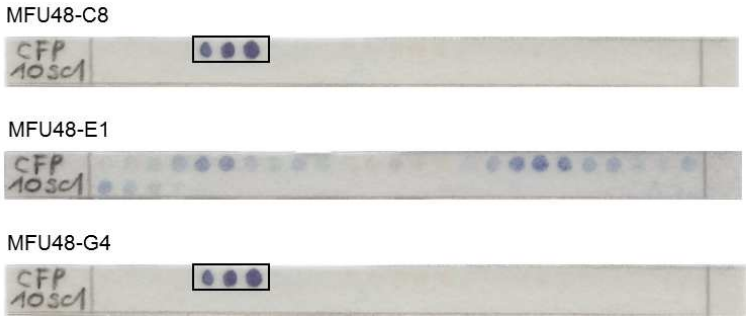


Figure 28: Epitope mapping of α-CFP-10 scFv-Fc with PepSpot membrane.
Membrane was incubated with respectively 5 µg scFv-Fc, detection of bound scFv-Fc with goat α-human IgG(Fc)-HRP, development with TMB. Immunostained spots are encircled.

Table 36: Epitope mapping of α-CFP-10 scFv-Fc, amino acid sequences of peptides on CFP-10 PepSpot membrane.
The immunostained spots and the identified epitope are highlighted.

spot no.	peptide
4	TLAQEAGNFERISGD
5	QEAGNFERISGDLKT
6	GNFERISGDLKTQID
7	ERISGDLKTQIDQVE
8	SGDLKTQIDQVESTA

The α-CFP-10 scFv-Fc were analysed by antigen titration ELISA (Figure 29). The antigen detection limit was determined to ~ 300 ng mL^{-1}.

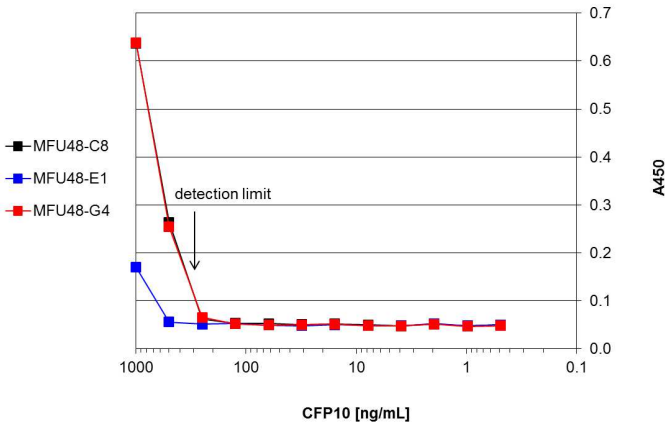


Figure 29: Antigen titration ELISA with α-CFP-10 scFv-Fc.
Different dilutions of antigen (CFP-10) were directly coated to the wells. Antigen detection with a scFv-Fc concentration of half maximal saturation, detection of bound scFv-Fc with goat α-human IgG (Fc)-HRP, development with TMB. Negative control BSA A_{450} = 0.05.

Recognition of Mtb and BCG cell extracts and culture filtrates was analysed by indirect ELISA (Figure 30). No reaction with the samples was detected.

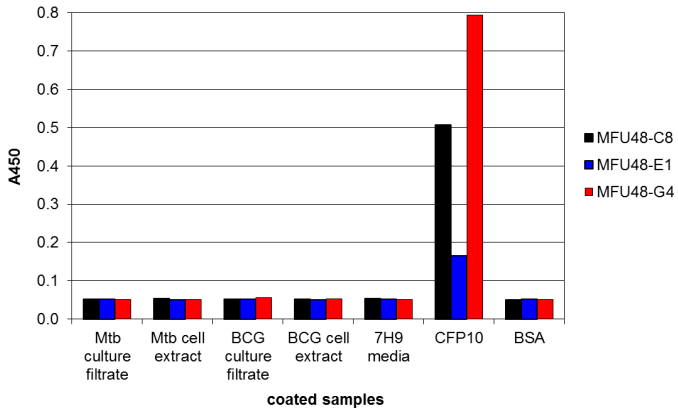


Figure 30: Reaction of α-CFP-10 scFv-Fc with Mtb/BCG cell extracts and culture filtrates determined by indirect ELISA.
Assay carried out as described in Figure 23.

A sandwich Lateral Flow Immuno Assay (LFIA) was attempted by Susanne Kämpfer (Lionex GmbH) with MFU48-E1 (epitope not distinguishable) as capture antibody and MFU48-C8 (epitope: ERISGDLKT) conjugated to colloidal gold as detection antibody. This LFIA only yielded un-reproducible results (data not shown). No signal was detected in another approach using α-CFP-10 scFv-Fc conjugated to HRP for detection in a sandwich ELISA (data not shown).

4.2.3 85 D

4.2.3.1 A-85 D scFv

Three individual 85 D binders were identified by panning HAL7/8 and subsequent screening ELISA followed by DNA sequencing (data not shown). The gene segments appearing in these α-85 D antibodies are outlined in Table 37.

Table 37: Comparison of heavy and light chain gene segments of α-85 D antibodies.

	heavy chain			light chain	
clone	**HV**	**D**	**HJ**	**LV**	**LJ**
MFU51-A6	IGHV1-46*01	IGHD3-10*01	IGHJ4*02	IGLV3-21*01	IGLJ3*01
MFU51-B10	IGHV3-9*01	IGHD3-16*01	IGHJ5*02	IGLV1-40*01	IGLJ3*02
MFU51-C2	IGHV3	IGHD6-19*01	IGHJ4*02	IGLV2-14*04	IGLJ3*02

All scFv were subcloned to the eukaryotic transient expression vector pCSE2.5-hIgG1-Fc-XP.

4.2.3.2 A-85 D scFv-Fc

HEK293-6E cells were transient transfected with the α-85 D scFv-Fc in pCSE2.5-hIgG1-Fc-XP. The scFv-Fc were purified from the culture supernatant via Protein A (with technical assistance of Franziska resch (TU-BS)). The protein yields were determined by a Lowry protein assay (Table 38). The obtained antibody solutions were analysed by SDS-PAGE followed by silver staining, α-human IgG(Fc) immunoblot and reducing gel analysis via Tape Station (Figure 31). A slight degradation of the Fc-part could be detected for MFU51-A6 by α-human IgG(Fc) immunoblot (Figure 31: B). This disassembly was visible in Tape Station analysis as well, issuing a five band pattern with 87.8 % purity for the target scFv-Fc (55 kDa) band (Figure 31: C). The other two α-85 D antibody purifications showed no sign of degradation, but additional protein bands of 204 and 231 kDa size.

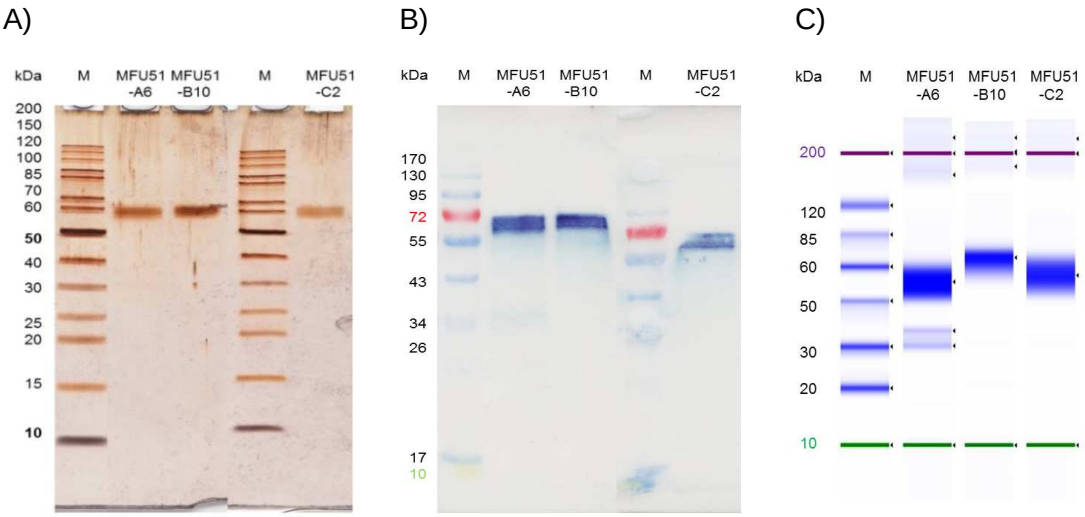


Figure 31: A) Silver staining, B) α-human IgG(Fc) immunoblot and C) Tape Station analysis of purified α-85 D scFv-Fc.
Analysis carried out as described in Figure 12. Results of two experiments joined for A) and B).

Table 38: Protein yields and purity of α-85 D scFv-Fc preparations.

scFv-Fc	yield [mg L^{-1}][a]	purity [%][b]
MFU51-A6	72.8	87.8
MFU51-B10	194.4	93.2
MFU51-C2	52.8	95.6

Analysis carried out as described in Table 35.

The antigen binding was analysed by titration ELISA (Figure 32). The antibodies bound 85 D specifically.

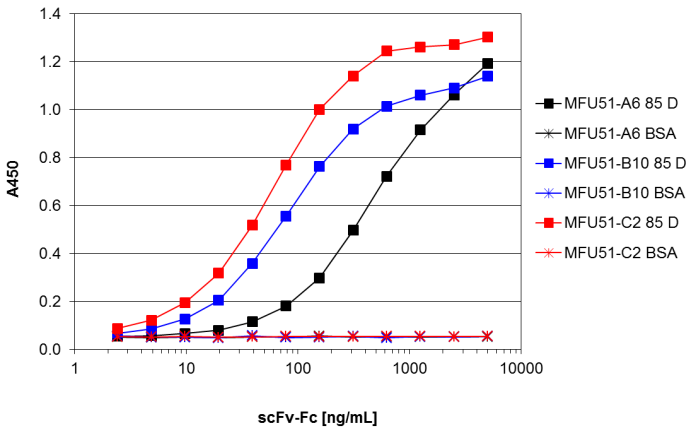


Figure 32: Titration ELISA of α-85 D scFv-Fc.
Dilution series of scFv-Fc were used for detection of directly coated antigen (85 D) or BSA (negative control), detection of bound scFv-Fc with goat α-human IgG(Fc)-HRP, development with TMB.

The cross reactivity with the other 85 complex antigens was analysed by ELISA (Figure 33) and immunoblot (Figure 34). It turned out, that MFU51-A6 and MFU51-C2 showed

cross reactivity with 85 A and 85 B. Whereas MFU51-B10 displayed no reactivity with the other 85 complex antigens neither in ELISA nor in immunoblot. A summary of the cross reactions is given in Table 39.

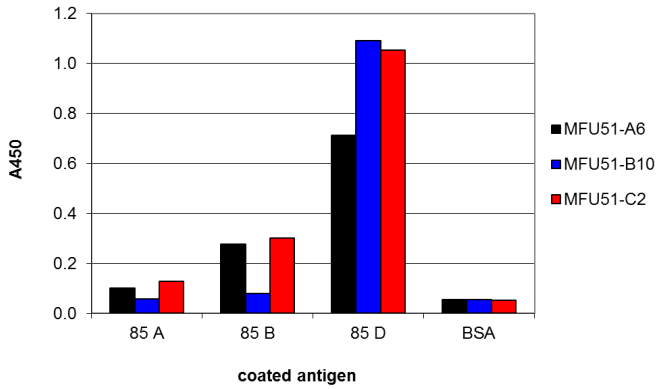


Figure 33: A-85 D indirect ELISA with α-85 D scFv-Fc, cross reactions with other 85 complex antigens.
100 ng of the antigens were directly coated to the wells. 85 D detection with α-85D scFv-Fc (using concentrations at half maximal saturation), detection of bound scFv-Fc with goat α-human IgG (Fc)-HRP, development with TMB.

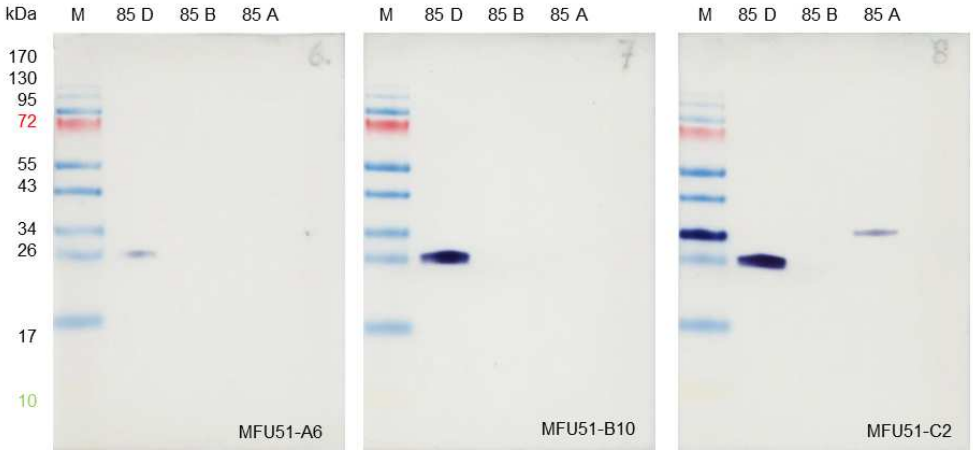


Figure 34: A-85 D immunoblot with α-85 D scFv-Fc, cross reactions with other 85 complex antigens.
500 ng of antigens 85 A, 85 B, 85 D and 7 µL of marker were separated on reducing 12 % SDS-PAGE and electro blotted to a PVDF membrane. After blocking, blots were incubated with respectively 5 µg scFv-Fc, bound scFv-Fc were detected using goat α-human IgG(Fc)-HRP, development with TMB.

Table 39: Cross reactions of α-85 D antibodies with other 85 complex antigens.

reaction with 85 complex antigens in ELISA				
clone	85 A	85 B	85 D	total
MFU51-A6	none	none	medium	none
MFU51-B10	none	none	strong	none
MFU51-C2	medium	none	strong	85 A
reaction with 85 complex antigens in immunoblot				
clone	85 A	85 B	85 D	total
MFU51-A6	weak	medium	strong	85 A, 85 B
MFU51-B10	none	none	strong	none
MFU51-C2	weak	medium	strong	85 A, 85 B
reaction with 85 complex antigens summary				
clone	85 A	85 B	85 D	total
MFU51-A6	immunoblot	immunoblot	immunoblot ELISA	85 A, 85 B
MFU51-B10	none	none	immunoblot, ELISA	none
MFU51-C2	immunoblot, ELISA	immunoblot	immunoblot, ELISA	85 A, 85 B

The reaction of the α-85 D antibodies with Mtb and BCG cell extracts and culture filtrates was analysed by indirect ELISA (Figure 35). A slight reaction with Mtb culture filtrate and Mtb/BCG cell extracts was discovered for MFU51-A6 and MFU51-B10.

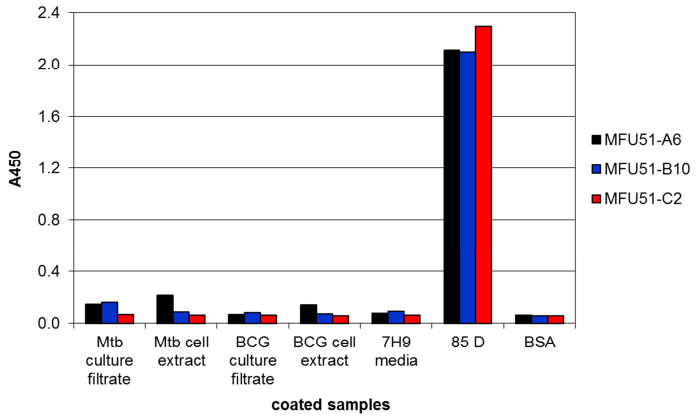


Figure 35: Reaction of α-85 D scFv-Fc with Mtb/BCG cell extracts and culture filtrates determined by indirect ELISA.
Assay carried out as described in Figure 23.

4.2.4 85 A

4.2.4.1 A-85 A scFv

Four individual 85 A binders were identified by panning HAL7/8 and subsequent screening ELISA followed by DNA sequencing (data not shown). All these antibodies have the same joining gene segment of the heavy chain (HJ, Table 40).

Table 40: Comparison of heavy and light chain gene segments of α-85 A antibodies.

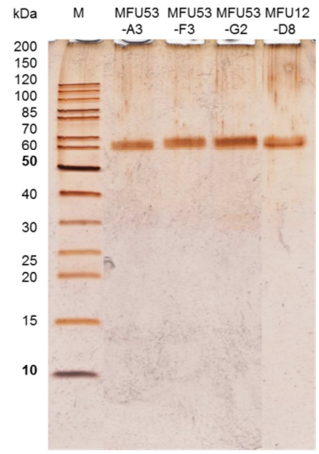
		heavy chain		light chain	
clone	**HV**	**D**	**HJ**	**LV**	**LJ**
MFU12-D8	IGHV3-33*01	IGHD5-5*01	IGHJ4*02	IGLV6-57*01	IGLJ3*01
MFU53-A3	IGHV3-23*04	IGHD6-25*01	IGHJ4*02	IGLV2-8*01	IGLJ3*01
MFU53-F3	IGHV1-2*02	IGHD6-19*01	IGHJ4*02	IGLV3-19*01	IGLJ3*01
MFU53-G2	IGHV3-30*03	IGHD3-22*01	IGHJ4*02	IGLV1-44*01	IGLJ3*02

All scFv were subcloned to the eukaryotic transient expression vector pCSE2.5-hIgG1-Fc-XP.

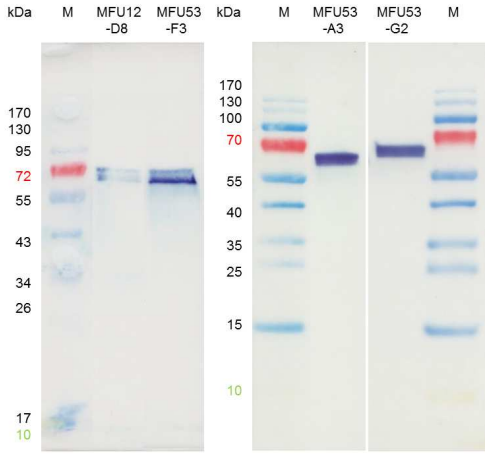
4.2.4.2 A-85 A scFv-Fc

HEK293-6E cells were transient transfected with the α-85 A scFv-Fc in pCSE2.5-hIgG1-Fc-XP. The scFv-Fc were purified from the culture supernatant via Protein A (with technical assistance of Franziska Resch (TU-BS)). The protein yield was determined by a Lowry protein assay (Table 41). The obtained antibody solutions were analysed by SDS-PAGE followed by silver staining, α-human IgG(Fc) immunoblot and reducing gel analysis via Tape Station. No degradation of the scFv-Fc was detected by either means (Figure 36). Tape Station analysis revealed one additional protein band per antibody preparation (except for MFU12-D8) issuing a purity of 97.8 – 100 % for the target scFv-Fc band (Table 41).

A)



B)



C)

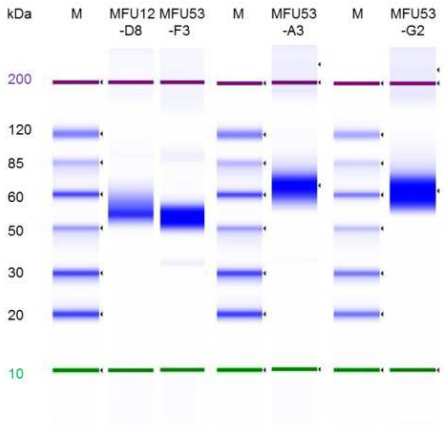


Figure 36: A) Silver staining, B) α-human IgG(Fc) immunoblot and C) Tape Station analysis of purified α-85 A scFv-Fc.
Analysis carried out as described in Figure 12. Results of three experiments joined for B) and C).

Table 41: Protein yield and purity of α-85 A scFv-Fc preparations.

scFv-Fc	yield[mg L^{-1}][a]	purity [%][b]
MFU12-D8	36.8	100
MFU53-A3	176.0	97.8
MFU53-F3	51.2	98.2
MFU53-G2	215.2	98.3

Analysis carried out as described in Table 35.

The antigen binding of the α-85 A scFv-Fc was analysed by titration ELISA (Figure 37). An unspecific reaction of MFU53-F3 with BSA, at a concentration higher than 200 ng mL^{-1}, was discovered. The other antibodies bound specifically to 85 A.

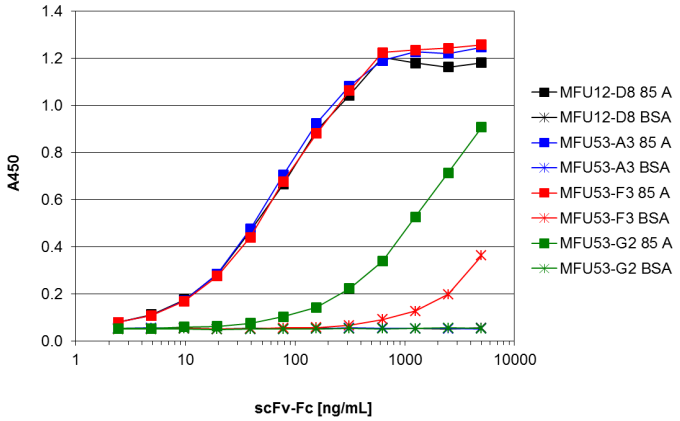


Figure 37: Titration ELISA of α-85 A scFv-Fc.
Dilution series of scFv-Fc were used for detection of directly coated antigen (85 A) or negative control (BSA), detection of bound scFv-Fc with goat α-human IgG(Fc)-HRP, development with TMB.

Epitope mapping of the α-85 A scFv-Fc was carried out on PepSpot membranes (Figure 38). MFU12-D8 reacted strong with the peptides 23 – 25, and weak with the peptides 22 and 26. In addition to that weak reactions with six other peptides were observed. Due to the tryptophan content of these peptides, which can lead to stickiness (S. Dübel, personal communication), the reactions were ignored for epitope determination. Through the overlap of the peptide sequences (22 – 26) the epitope was determined to “SPA” (aa 76 – 78 of antigen 85 A) (Table 42). MFU53-A3 reacted strong with the peptides 24 – 27 and hence it was concluded it recognized “LYLLDG” (aa 79 – 84), which is directly preceded by “SPA” in the antigen sequence. The antigenic determinant of MFU53-G2 was identified to “AFSRPGLPV” (aa 43 – 51), due to weak reactions with the peptides 13 – 15. The epitope of MFU53-F3 was not distinguishable because of the variety of reactions with the PepSpots, which indicated unspecificity or a conformational epitope.

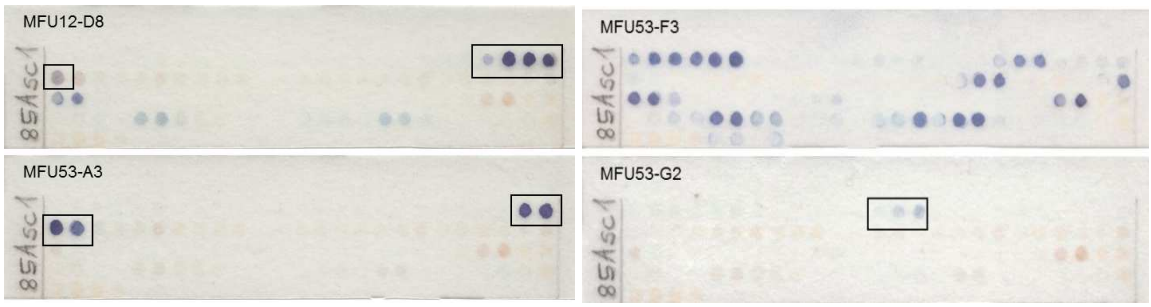


Figure 38: Epitope mapping of α-85 A scFv-Fc with PepSpot membrane.
Membrane was incubated with respectively 5 µg scFv-Fc (except for MFU53-F3, only 1 µg), detection of bound scFv-Fc with goat α-human IgG (Fc)-HRP, development with TMB. Immunostained spots are encircled. No cross reaction of the secondary antibody with the PepSpots was determined (data not shown).

Table 42: Epitope mapping of α-85 A scFv-Fc, amino acid sequences of peptides on 85 A PepSpot membrane.
The immunostained spots and the identified epitopes are highlighted.

clone	spot no.	peptide
MFU12-D8	21	MGRDIKVQFQSGGAN
	22	DIKVQFQSGGANSPA
	23	VQFQSGGANSPALYL
	24	QSGGANSPALYLLDG
	25	GANSPALYLLDGLRA
	26	SPALYLLDGLRAQDD
	27	LYLLDGLRAQDDFSG
MFU53-A3	23	VQFQSGGANSPALYL
	24	QSGGANSPALYLLDG
	25	GANSPALYLLDGLRA
	26	SPALYLLDGLRAQDD
	27	LYLLDGLRAQDDFSG
	28	LDGLRAQDDFSGWDI
MFU53-G2	12	AVGGTATAGAFSRPG
	13	GTATAGAFSRPGLPV
	14	TAGAFSRPGLPVEYL
	15	AFSRPGLPVEYLQVP
	16	RPGLPVEYLQVPSPS

The crystal structure of antigen 85 A was determined by (Ronning *et al.*, 2004). Therefore it was possible to visualize the determined epitopes on the three-dimensional (3D) structure of the antigen (Figure 39). The distance between epitope region “LYLLDG” and “AFSRGPLV” was computed to ~ 2.5 nm. Considering the width of an antibody (~ 4 nm, (Boehm *et al.*, 1999) sandwich detection of an 85 A monomer with the investigated scFv-Fc was expected to be difficult.

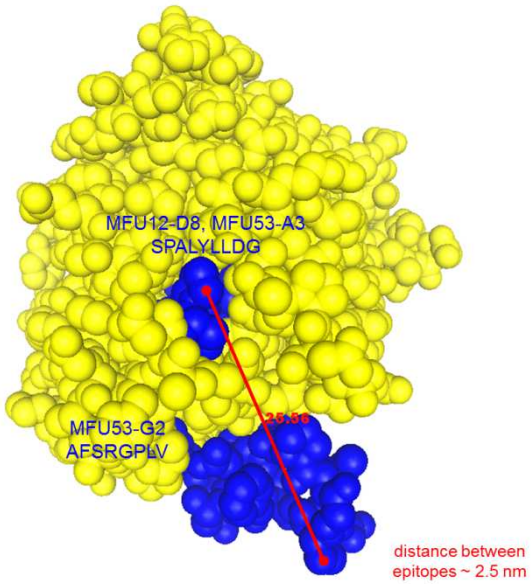


Figure 39: 3D structure of antigen 85 A, epitopes of α-85 A antibodies.
Pdb1sfr (Resolution 2.7 Å,(Ronning *et al.*, 2004) was modified with 3D molecule viewer (Invitrogen). Only protein chain A is shown, atoms are displayed as space filling balls, epitopes are marked in blue, distance measurement in red.

Sequence comparison of the epitopes of the α-85 A antibodies with corresponding sequences of the other 85 complex antigens provided information about possible cross reactions (Table 43: A). The complete epitope “AFSRPGPLV” is present in antigen 85 B and 85 C, not in 85 D. Homologous regions of the epitope region “SPALYL(LDG)” can be found in 85 B, C, and D. Experimental study of this cross reactions was carried out by indirect ELISA (Figure 40) and immunoblot (Figure 41). It was discovered that not all expected cross reactions actually came to pass. Only a very weak cross reaction of MFU53-A3 with 85 D was detected by immunoblot, whereupon additional cross reaction with 85 B was expected. Strong cross reactivity with 85 B in ELISA and immunoblot was observed for MFU53-F3. MFU53-G2 displayed a weak reaction with 85 B in ELISA and no reaction with 85 D, as predicted by sequence comparison. Despite the existence of the entire MFU12-D8 epitope on the 85 B sequence, no cross reaction was observed. A summary of the expected cross reactions in comparison to determined cross reactions is given in Table 43.

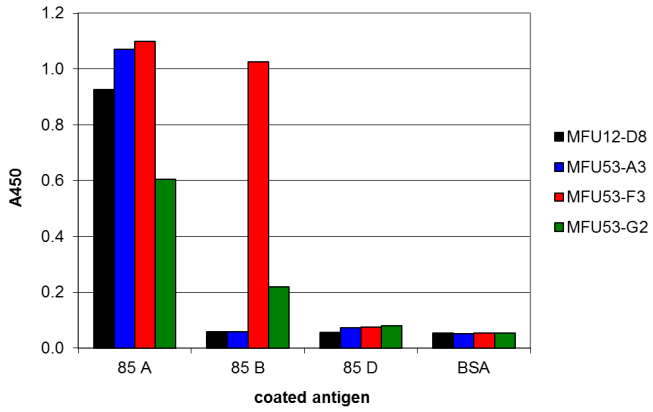


Figure 40: A-85 A indirect ELISA with α-85 A scFv-Fc, cross reactions with other 85 complex antigens.
Assay carried out as described in Figure 33.

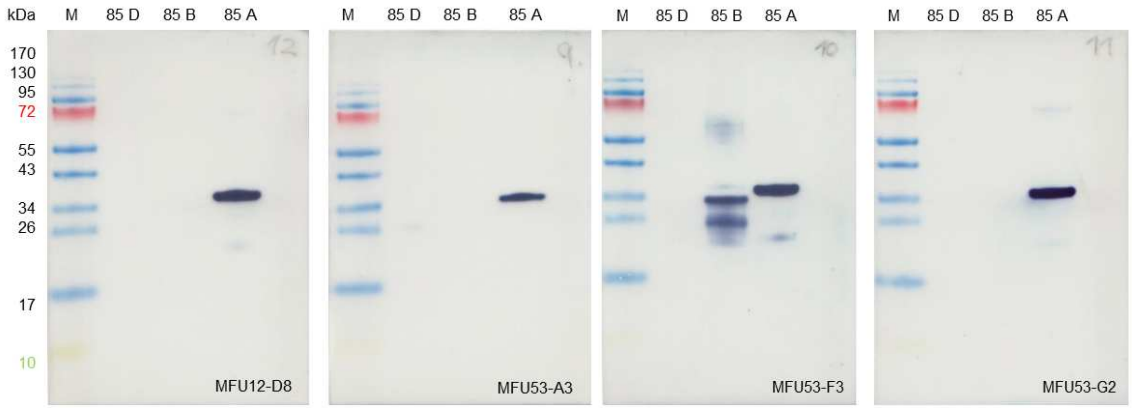


Figure 41: A-85 A immunoblot with α-85 A scFv-Fc, cross reactions with other 85 complex antigens.
Assay carried out as described in Figure 34.

Table 43: Cross reactions of α-85 A antibodies with other 85 complex antigens.

A) sequence comparison (corresponding sequences to α-85 A epitopes)					
clone	85 A	85 B	85 C*	85 D	expected cross reaction
MFU12-D8	SPA	SPA	PHA	PHA	85 B
MFU53-A3	LYLLDG	VYLLDG	VYLLDG	VYLLDA	85 B, 85 C, 85 D
MFU53-F3	?	?	?	?	85 B, 85 C, 85 D
MFU53-G2	AFSRPGLPV	AFSRPGLPV	AFSRPGLPV	-	85 B, 85 C
B) reaction with 85 complex antigens in ELISA					
clone	85 A	85 B	85 C*	85 D	determined cross reaction
MFU12-D8	strong	none	-	none	none
MFU53-A3	strong	none	-	none	none
MFU53-F3	strong	strong	-	none	85 B
MFU53-G2	strong	weak	-	none	weak 85 B
C) reaction with 85 complex antigens in immunoblot					
clone	85 A	85 B	85 C*	85 D	determined cross reaction
MFU12-D8	strong	none	-	none	none
MFU53-A3	strong	none	-	weak	weak 85 D
MFU53-F3	strong	strong	-	none	85 B
MFU53-G2	strong	none	-	none	none
D) reaction with 85 complex antigens summary					
clone	85 A	85 B	85 C*	85 D	determined cross reaction
MFU12-D8	western, ELISA	none	-	none	none
MFU53-A3	western, ELISA	none	-	western	85 D
MFU53-F3	western, ELISA	western, ELISA	-	none	85 B
MFU53-G2	western, ELISA	ELISA	-	none	85 B

*Purified antigen 85 C was not available for immunological assays, nevertheless it is listed for completeness of the illustration

The antigen detection limits of the α-85 A scFv-Fc were determined to ~ 10 ng mL^{-1} for MFU12-D8 and MFU53-A3, and ~ 60 ng mL^{-1} for MFU53-F3 and MFU53-G2 by antigen titration ELISA (Figure 42).

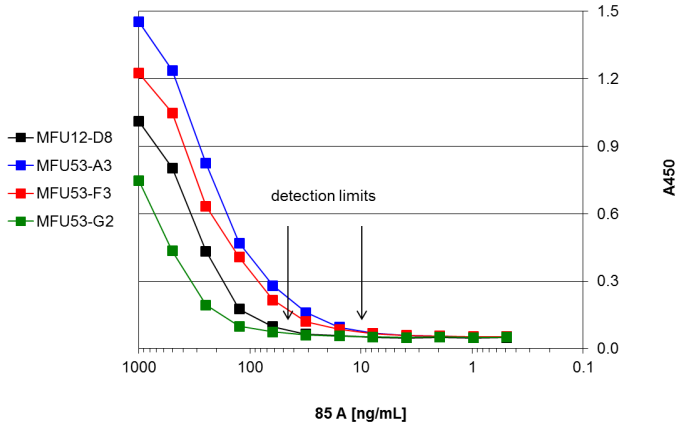


Figure 42: Antigen titration ELISA with α-85 A scFv-Fc.
Different dilutions of antigen (85 A) were directly coated to the wells. Antigen detection with a scFv-Fc concentration of half maximal saturation, detection of bound scFv-Fc with goat α-human IgG(Fc)-HRP, development with TMB. Negative control BSA A_{450} = 0.05.

The reaction of the α-85 A antibodies with Mtb and BCG cell extracts and culture filtrates was analysed by indirect ELISA (Figure 43). No specific reaction with Mtb/BCG cell extracts or culture filtrates was detected.

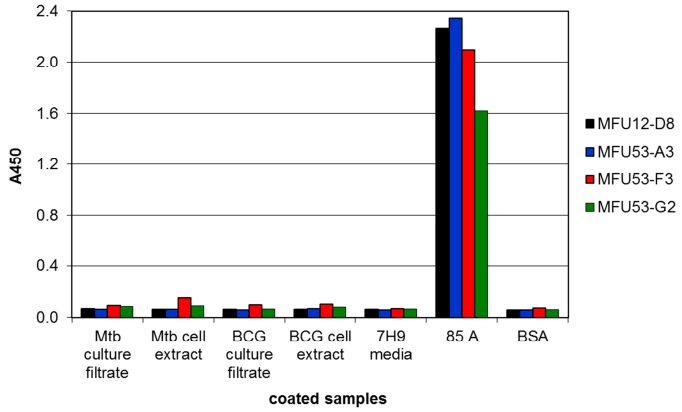


Figure 43: Reaction of α-85 A scFv-Fc with Mtb/BCG cell extracts and culture filtrates determined by indirect ELISA.
Assay carried out as described in Figure 23.

4.2.4.3 A-85 A scFv-Fc-HRP

Visualization of the α-85 A antibody epitopes on the 3D structure of the antigen revealed an unfavourable position for sandwich detection. Considering that (Ronning *et al.*, 2004) discovered a trimeric structure for antigen 85 A, a sandwich assay was attempted nevertheless. Therefore MFU53-A3 scFv-Fc (recognizing "LYLLDG") and MFU53-G2 scFv-Fc (recognizing "AFSRGPLV") were conjugated to HRP. The necessary dilution of the antibody-HRP conjugates was determined by direct ELISA (data not shown) to 0.6 µg mL^{-1} for MFU53-G2 and 0.07 µg mL^{-1} for MFU53-A3. The cross reactivity of the achieved antibody-HRP conjugates was analysed by α-85 A immunoblot and α-85 A direct ELISA. No cross reactions were detected through immunoblot (exemplary displayed for MFU53-A3-HRP in Figure 44). Slight cross reactions with 85 B were detected by ELISA (exemplary displayed for MFU53-A3-HRP in Figure 47).

Figure 44: Cross reactions of α-85 A MFU53-A3 scFv-Fc-HRP in immunoblot.
Blot preparation as described in Figure 24. After blocking, membrane was incubated with MFU53-A3 scFv-Fc-HRP at a concentration of ~ 0.2 µg mL^{-1}, development with TMB.

Sandwich detection of the antigen was attempted with all four α-85 A scFv-Fc as capture antibodies and MFU53-A3/G2-HRP as detection antibodies. Sandwich detection of the recombinant antigen was successful with all combinations, offering a detection limit of ~ 125 ng mL^{-1} (exemplary displayed for MFU53-A3-HRP detection in Figure 45).

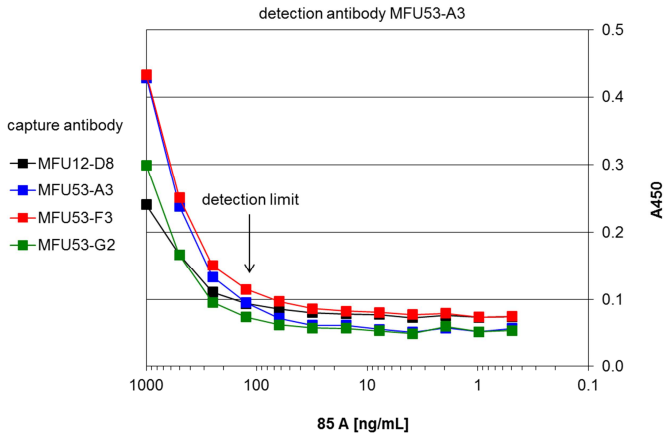


Figure 45: Sandwich 85 A (07-1/1) titration ELISA with α-85 A scFv-Fc.
100 ng of capture scFv-Fc were directly coated to the wells, after blocking different dilutions of antigen 85 A (batch 07-1/1) were applied and incubated, detection of bound antigen with MFU53-A3-scFv-Fc-HRP at ~ 0.07 µg mL^{-1}, development with TMB. Negative control BSA A_{450} = 0.08.

Capturing with MFU53-A3 and simultaneous detection with MFU53-A3-HRP was possible (the same was observed for MFU53-G2), which indicated the detection of antigenic associates. There was not enough sample of the antigen batch (07-1/1) left to investigate the aggregation behavior. But another 85 A batch (12-2/1) was available and was analysed using analytical SEC.

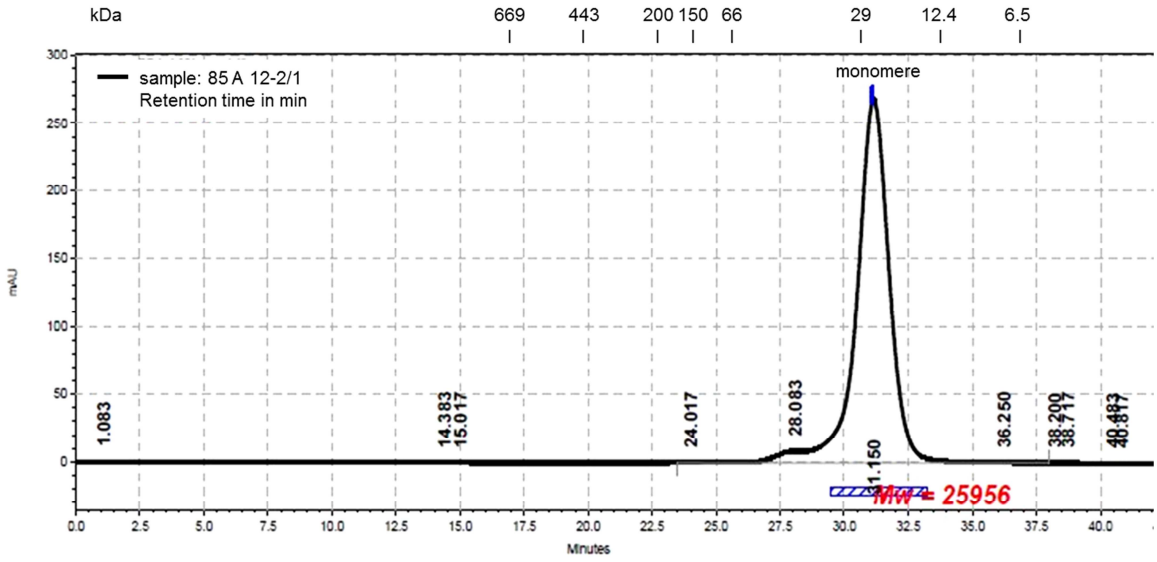


Figure 46: Analytical SEC of antigen 85 A (batch 12-2/1).
500 µL of sample were applied to Superdex 200 10/300 GL column, running buffer was PBS pH 7.4. Standards: 1. 669 kDa 17 min, 2. 443 kDa 20 min, 3. 200 kDa 23 min, 4. 150 kDa 24 min, 5. 66 kDa 26 min, 6. 29 kDa 31 min, 7. 12.4 kDa 34 min, 8. 6.5 kDa 37 min. Analysis was performed with technical assistance of Wiebke Prilop (Lionex GmbH).

The theoretical molecular mass of an 85 A monomer (recombinant with N-terminal His-tag and without the signal sequence) was computed with the ExPASy prot param tool to 32.6 kDa. A dominant peak at 31.2 kDa was visible in the chromatogram of the analytical SEC (Figure 46). Based on this result 85 A (batch 12-1/1 in PBS pH 7.4) was considered to be mainly monomeric. A sandwich ELISA assay with both antigen batches in comparison offered a ~ 50 % greater reaction with batch 07-1/1 than with batch 12-1/1 (Figure 47). Using only one antibody for capturing and detection was possible with batch 12-1/1 as well, indicating the presence of associates in this antigen preparation.

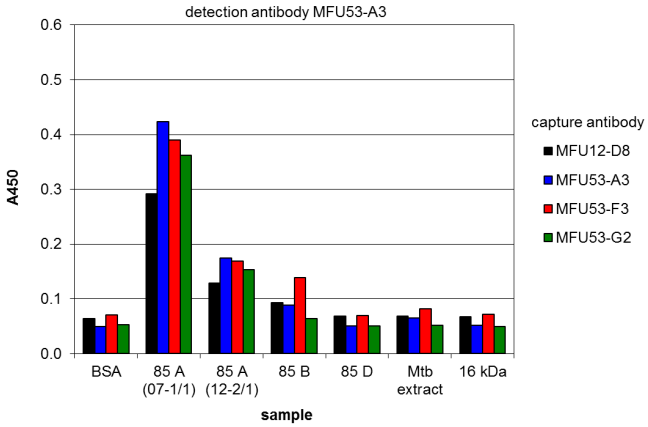


Figure 47: A-85 A sandwich ELISA with α-85 A scFv-Fc, recognition of multimers, cross reactions.
100 ng of capture scFv-Fc were directly coated to the wells, after blocking 100 ng of the samples were applied and incubated, detection of bound antigen with MFU53-A3-scFv-Fc-HRP at ~ 0.07 µg mL^{-1}, development with TMB.

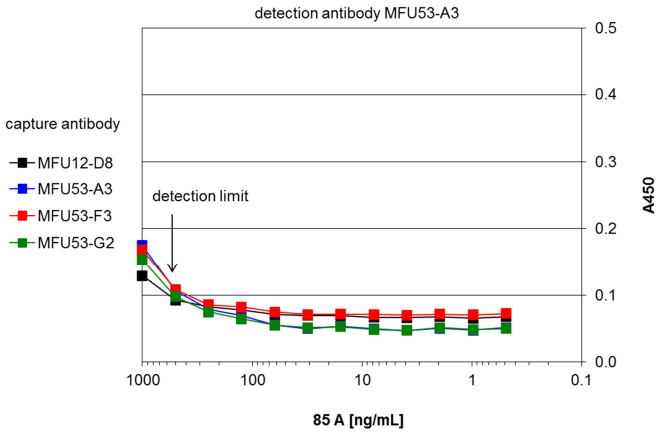


Figure 48: Sandwich 85 A (12-1/1) titration ELISA with α-85 A scFv-Fc.
Assay performed as described in Figure 48, but with 85 A batch 12-1/1.

By antigen titration of batch 12-1/1 a detection limit of ~ 500 ng mL^{-1} was determined, which was four times less sensitive than with batch 07-1/1. Assuming that batch 07-1/1

contained a greater amount of multimers, sandwich detection of 85 A was benefitted by antigenic associates. Regarding the obtained data, a final statement if the α-85 A sandwich ELISA recognized only associates was not possible.

4.2.4.4 A-85 A scFv-Fc-gold

A-85 A scFv-Fc were conjugated to colloidal gold by Lionex GmbH. The obtained scFv-Fc-gold preparations reacted with the antigen in a direct assay. Sandwich assays with different antibody combinations were performed, delivering only unspecific results (data not shown).

4.2.5 85 B

4.2.5.1 A-85 B scFv

After panning HAL7/8 for α- 85 B scFv, 92 clones from the third panning round were produced in MTP. Their ability to bind 85 B was analysed by screening ELISA, resulting in 51 signals higher than A_{450} = 0.1 (Figure 49). From 11 of these clones plasmid DNA was isolated and sent for DNA-sequencing.

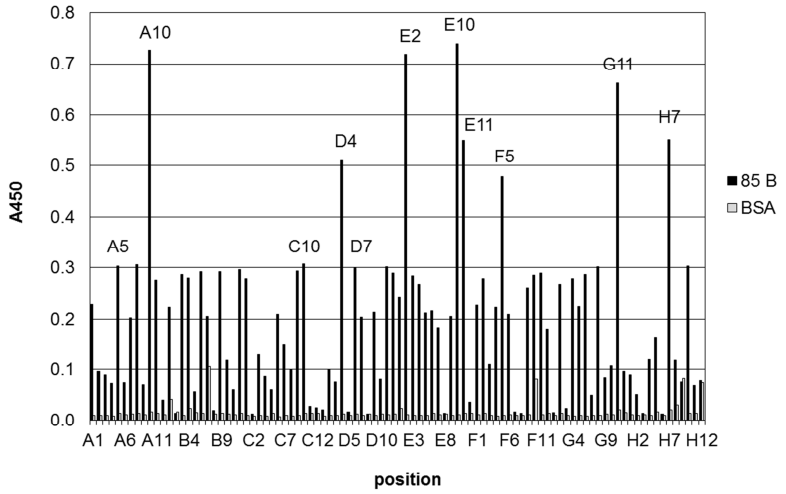


Figure 49: Screening ELISA for 85 B binding scFv in HAL7/8.
Culture supernatants containing soluble scFv of 92 single clones (3. panning round) were screened for their ability to bind antigen (85 B) and BSA (negative control). Detection of bound scFv with mouse α-c-Myc-tag 9E10 IgG followed by goat α-mouse IgG(Fc)-HRP, development with TMB.

In the end five individual 85 B binders were identified (Table 44). All α-85 B antibodies contain a variable gene segment of the heavy chain (HV) of subfamily V_H3. Four binders have a lambda light chain ($V_L\lambda$), one has a kappa light chain ($V_L\kappa$). All $V_L\lambda$ clones have a joining segment of the heavy chain (HJ) of subfamily J4, a joining segment of the light chain (LJ) of subfamily J3, and a variable gene segment of the heavy chain (HV) of subfamily V_H3 gene 30.

Table 44: Comparison of heavy and light chain gene segments of α-85 B antibodies.

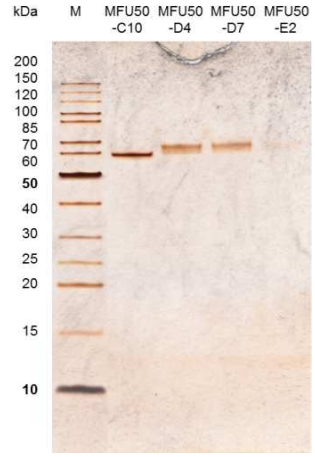
	heavy chain			light chain	
clone	**HV**	**D**	**HJ**	**LV**	**LJ**
MFU50-A10	IGHV3-30*04	IGHD6-19*01	IGHJ4*02	IGLV8-61*01	IGLJ3*02
MFU50-C10	IGHV3	IGHD2-15*01	IGHJ6*02	IGKV1-12*01	IGKJ2*01
MFU50-D4	IGHV3-30*18	IGHD3-3*02	IGHJ4*02	IGLV2-14*01	IGLJ3*01
MFU50-D7	IGHV3-30*04	IGHD4-17*01	IGHJ4*02	IGLV3-21*02	IGLJ3*02
MFU50-E2	IGHV3-30*03	IGHD6-13*01	IGHJ4*02	IGLV7-43*01	IGLJ3*02

All scFv were subcloned to the eukaryotic transient expression vector pCSE2.5-hIgG1-Fc-XP.

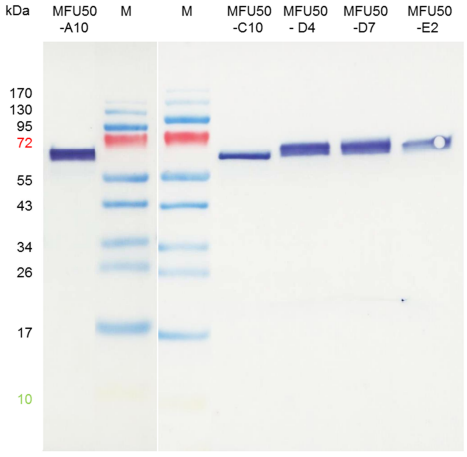
4.2.5.2 A-85 B scFv-Fc

HEK293-6E cells were transient transfected with the α-85 B scFv-Fc in pCSE2.5-hIgG1-Fc-XP. The scFv-Fc were purified from the culture supernatant via Protein A (with technical assistance of Franziska Resch (TU-BS)). The protein yield was determined by a Lowry protein assay (Table 45). No degradation could be detected by silver-stained SDS-PAGE (Figure 50: A), α-human IgG(Fc) immunoblot (Figure 50: B) or reducing gel analysis via Tape Station (Figure 50: C). Up to three very faint additional protein bands with a higher molecular mass than the target scFv-Fc were detected by Tape Station, issuing a purity of 93.4 – 96.9 % for the antibody preparations (Table 45).

A)



B)



C)

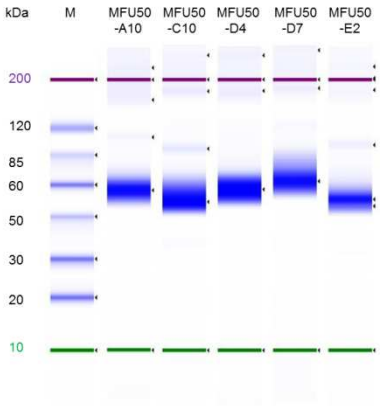


Figure 50: A) Silver staining, B) α-human IgG(Fc) immunoblot and C) Tape Station analysis of purified α-85 B scFv-Fc.
Analysis carried out as described in Figure 12. A) silver staining of MFU50-A10 scFv-Fc is missing, B) results of two experiments joined.

Table 45: Protein yields and purity of α-85 B scFv-Fc preparations.

scFv-Fc	yield [mg L^{-1}][a]	purity [%][b]
MFU50-A10	198.4	94.4
MFU50-C10	196.8	94.8
MFU50-D4	194.4	96.9
MFU50-D7	183.2	96.4
MFU50-E2	50.4	93.4*

Analysis carried out as described in Table 35. *53 + 54 kDa values combined*

Antigen binding of the α-85 B scFv-Fc was analysed by titration ELISA (Figure 51). All antibodies bound 85 B specifically. Further, all α-85 B antibodies were binding to continuous sequences determined by immunoblot (data not shown).

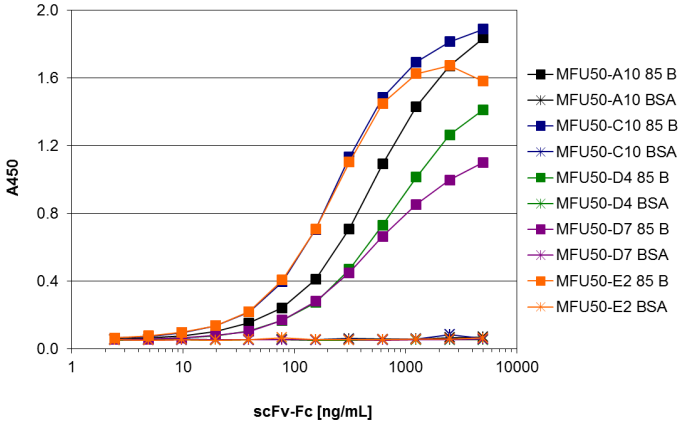


Figure 51: Titration ELISA of α-85 B scFv-Fc.
Dilution series of scFv-Fc were used for detection of directly coated antigen (85 B) or BSA (negative control), detection of bound scFv-Fc with goat α-human IgG(Fc)-HRP, development with TMB.

Epitope mapping of the α-85 B scFv-Fc was carried out on PepSpot membranes (Figure 52). Through the overlap of the peptide sequences the epitopes were determined (Table 46). It was observed that MFU50-D7 and MFU50-D4 recognized the same region of the antigen “AFSRPGLPV(EYL)” (aa 39 – 51 respectively aa 39 – 47). Similarly the epitope of MFU50-A10 and MFU50-E2 was identified to “SSDPAWERN(DPT) “(aa 223 – 234 respectively aa 223 – 231). MFU50-C10 reacted with a third antigenic determinant “SPAVYL” (aa 73 – 78). Interestingly corresponding sequences of the epitope regions “AFSRGPLVEYL” and “SPAVYL” were recognized by the α-85 A antibodies as well.

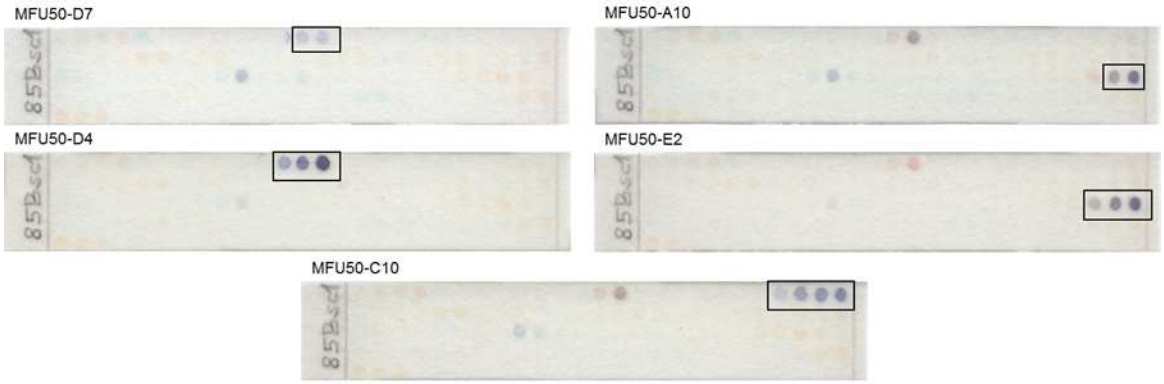


Figure 52: Epitope mapping of α-85 B scFv-Fc with PepSpot membrane.
Membrane was incubated with respectively 5 µg scFv-Fc, detection of bound scFv-Fc with goat α-human IgG(Fc)-HRP, development with TMB. Immunostained spots are encircled. No cross reaction of the secondary antibody with the PepSpots was determined (data not shown).

Table 46: Epitope mapping of α-85 B scFv-Fc, amino acid sequences of peptides on 85 B PepSpot membrane.
The immunostained spots and the identified epitopes are highlighted.

clone	spot no.	peptide
MFU50-D7	12	GAATAGAFSRPGLPV
	13	TAGAFSRPGLPVEYL
	14	AFSRPGLPVEYLQVP
	15	RPGLPVEYLQVPSPS
MFU50-D4	11	LAGGAATAGAFSRPG
	12	GAATAGAFSRPGLPV
	13	TAGAFSRPGLPVEYL
	14	AFSRPGLPVEYLQVP
	15	RPGLPVEYLQVPSPS
MFU50-A10	73	ADMWGPSSDPAWERN
	74	WGPSSDPAWERNDPT
	75	SSDPAWERNDPTQQI
	76	PAWERNDPTQQIPKL
MFU50-E2	72	YKAADMWGPSSDPAW
	73	ADMWGPSSDPAWERN
	74	WGPSSDPAWERNDPT
	75	SSDPAWERNDPTQQI
	76	PAWERNDPTQQIPKL
MFU50-C10	21	DIKVQFQSGGNNSPA
	22	VQFQSGGNNSPAVYL
	23	QSGGNNSPAVYLLDG
	24	GNNSPAVYLLDGLRA
	25	SPAVYLLDGLRAQDD
	26	VYLLDGLRAQDDYNG

The crystal structure of antigen 85 B was determined by Anderson and colleagues (Anderson *et al.*, 2001). Therefore it was possible to visualize the determined epitopes on the 3D structure of the antigen (Figure 53). The distance between epitope regions "AFSRGPLV(EYL)" and "SPAVYL" was computed to ~ 2.5 nm, between "SPAVYL" and "SSDPAWERN(DPT)" to ~ 4 nm and between "SSDPAWERN(DPT)" and "AFSRGPLV(EYL)" to ~ 5 nm. Considering the width of an antibody (~ 4 nm, (Boehm *et*

al., 1999)), sandwich detection was likely targeting epitopes on different sites of the antigen.

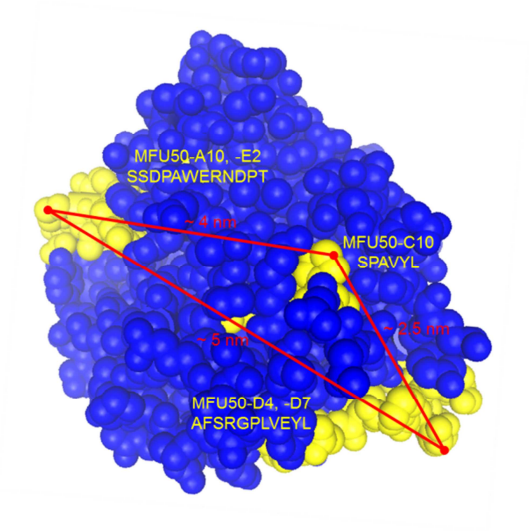


Figure 53: 3D structure of antigen 85 B, epitopes of α-85 B antibodies.
Pdb1f0n (Resolution 1.9 Å, (Anderson *et al.*, 2001) was modified with 3D molecule viewer (Invitrogen). Only protein chain A is shown, atoms are displayed as space filling balls, epitopes are marked in yellow, distance measurement in red.

Sequence comparison of the epitopes of the α-85 B antibodies with corresponding sequences of the other 85 complex antigens provided information about possible cross reactions (Table 47: A). The complete epitope “AFSRPGPLV(EYL)” is present in antigen 85 A and 85 C, not in 85 D. Homologous regions of the epitopes “SPAVYL” and “SSDPAWERN(DPT)” can be found in 85 A, C, and D. Experimental study of 85 complex cross reactivity was carried out by indirect ELISA (Figure 54) and immunoblot (Figure 55). MFU50-A10 reacted with 85 A and 85 D in ELISA and immunoblot, and MFU50-D4 displayed a weak cross reactivity with 85 A in ELISA as predicted. MFU50-E2 bound antigen 85 B weakly and showed no cross reactions. MFU50-D7 reacted weakly with 85 B in ELISA and strong in immunoblot, no reaction with the other 85 complex antigens was detected by either method. MFU50-C10 was not cross reactive as well. A summary of the expected cross reactions in comparison to determined cross reactions is outlined in Table 47.

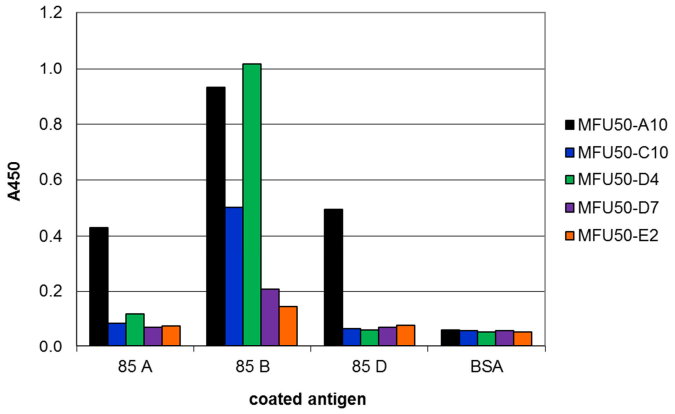


Figure 54: A-85 B indirect ELISA with α-85 B scFv-Fc, cross reactions with other 85 complex antigens.
Assay carried out as described in Figure 33.

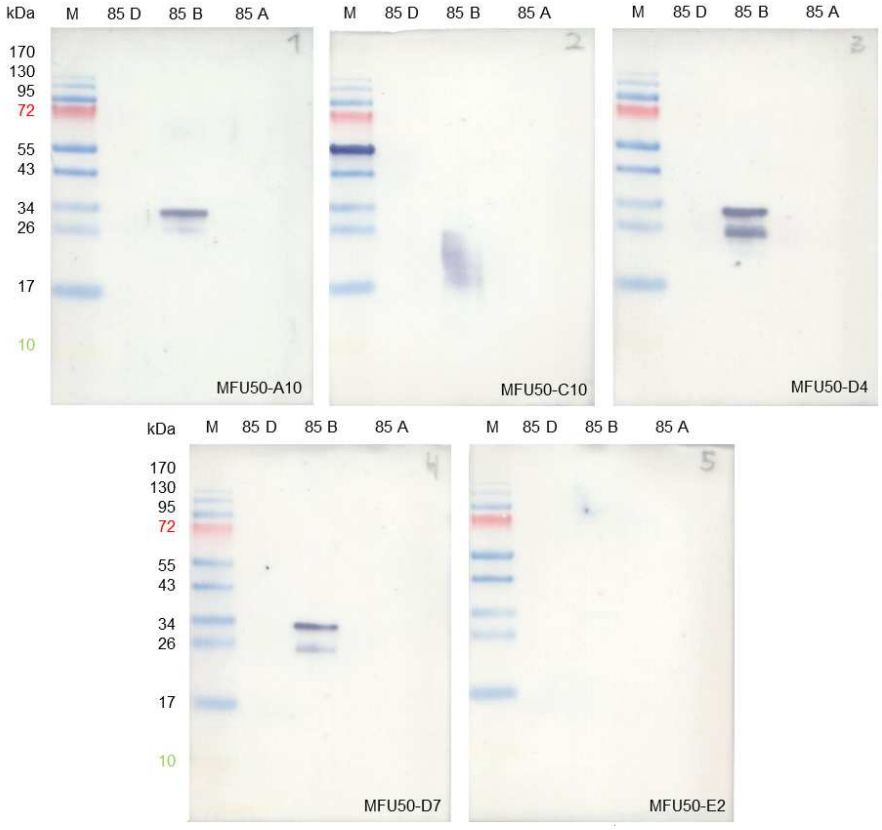


Figure 55: A-85 B immunoblot with α-85 B scFv-Fc, cross reactions with other 85 complex antigens.
Assay carried out as described in Figure 34.

Table 47: Cross reactions of α-85 B antibodies with other 85 complex antigens.

A) sequence comparison (corresponding sequences to α-85 B epitopes)					
clone	85 A	85 B	85 C*	85 D	expected cross reaction
MFU50-A10	KEDPAW QRNDPL	SSDPAW ERNDPT	SSDPAW KRNDPM	ASDPAA	85 A, 85 C, 85 D
MFU50-C10	SPALYL	SPAVYL	PHAVYL	PHAVYL	85 A, 85 C, 85 D
MFU50-D4	AFSRPG LPV	AFSRPG LPV	AFSRPG LPV	-	85 A, 85 C
MFU50-D7	AFSRPG LPVEYL	AFSRPG LPVEYL	AFSRPG LPVEYL	-	85 A, 85 C
MFU50-E2	KEDPAW QRN	SSDPAW ERN	SSDPAW KRN	ASDPAA	85 A, 85 C, 85 D
B) reaction with 85 complex antigens in ELISA					
clone	85 A	85 B	85 C*	85 D	determined cross reaction
MFU50-A10	strong	strong	-	strong	85 A, 85 D
MFU50-C10	none	strong	-	none	none
MFU50-D4	weak	strong	-	none	weak 85 A
MFU50-D7	none	weak	-	none	none
MFU50-E2	none	weak	-	none	none
C) reaction with 85 complex antigens in immunoblot					
clone	85 A	85 B	85 C*	85 D	determined cross reaction
MFU50-A10	weak	strong	-	weak	85 A, 85 D
MFU50-C10	none	strong	-	none	none
MFU50-D4	none	strong	-	none	none
MFU50-D7	none	strong	-	none	none
MFU50-E2	none	weak	-	none	none
D) reaction with 85 complex antigens summary					
clone	85 A	85 B	85 C*	85 D	determined cross reaction
MFU50-A10	western, ELISA	western, ELISA	-	western, ELISA	85 A, 85 D
MFU50-C10	none	western, ELISA	-	none	none
MFU50-D4	ELISA	western, ELISA	-	none	85 A
MFU50-D7	none	western, ELISA	-	none	none
MFU50-E2	none	western, ELISA	-	none	none

*Purified antigen 85 C was not available for immunological assays, nevertheless it is listed for completeness of the illustration

The antigen detection limits of the α-85 B scFv-Fc were determined by antigen titration ELISA (Figure 56). About 5 ng mL^{-1} were detected by MFU50-C10, ~ 10 ng mL^{-1} by MFU50-A10 and MFU50-E2, and ~ 30 ng mL^{-1} by MFU50-D4 and MFU50-D7.

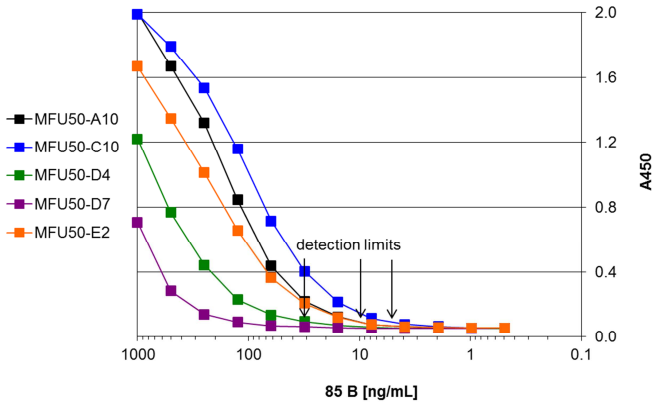


Figure 56: Antigen titration ELISA with α-85 B scFv-Fc.
Different dilutions of antigen (85 B) were directly coated to the wells. Antigen detection with a scFv-Fc concentration of half maximal saturation, detection of bound scFv-Fc with goat α-human IgG(Fc)-HRP, development with TMB. Negative control BSA A_{450} = 0.05.

The α-85 B antibody binding to Mtb/BCG cell extracts and culture filtrates was analysed by indirect ELISA (Figure 57). In this assay only MFU50-A10 reacted with Mtb culture filtrate and Mtb/BCG cell extracts. The other antibodies bound merely 85 B. A weak cross reaction of MFU50-A10 with 7H9 medium was detected.

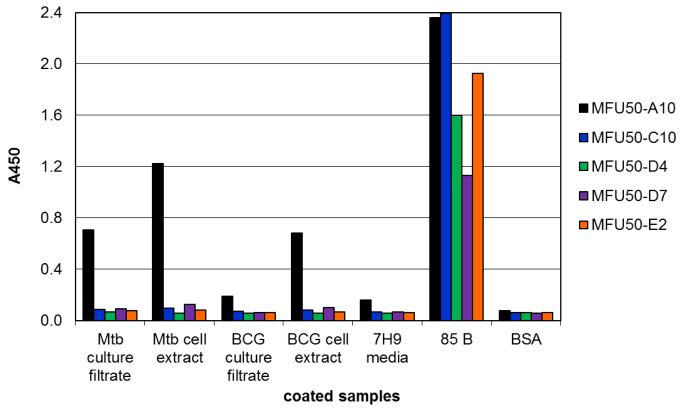


Figure 57: Reaction of α-85 B scFv-Fc with Mtb/BCG cell extracts and culture filtrates determined by indirect ELISA.
Assay carried out as described in Figure 23.

4.2.5.3 A-85 B sandwich ELISA

Due to the availability of five different antibodies with three different epitopes on the target antigen a sandwich α-85 B assay was attempted. MFU50-A10 (epitope "SSDPAWERNDPT"), MFU50-C10 (epitope "SPAVYL") and MFU50-D4 (epitope "AFSRPGLPV") scFv-Fc were conjugated to HRP. The necessary dilutions of the antibody-HRP conjugates were determined by direct ELISA (data not shown) to ~ 0.05 µg mL^{-1} for all preparations. Sandwich detection of the antigen was attempted

with MFU50-A10-HRP, MFU50-C10-HRP or MFU50-D4-HRP as detection antibodies and capturing was carried out with corresponding antibodies recognizing different epitopes (than the detection antibody) on the target. Sandwich detection of the recombinant antigen was successful with all combinations (Figure 58).

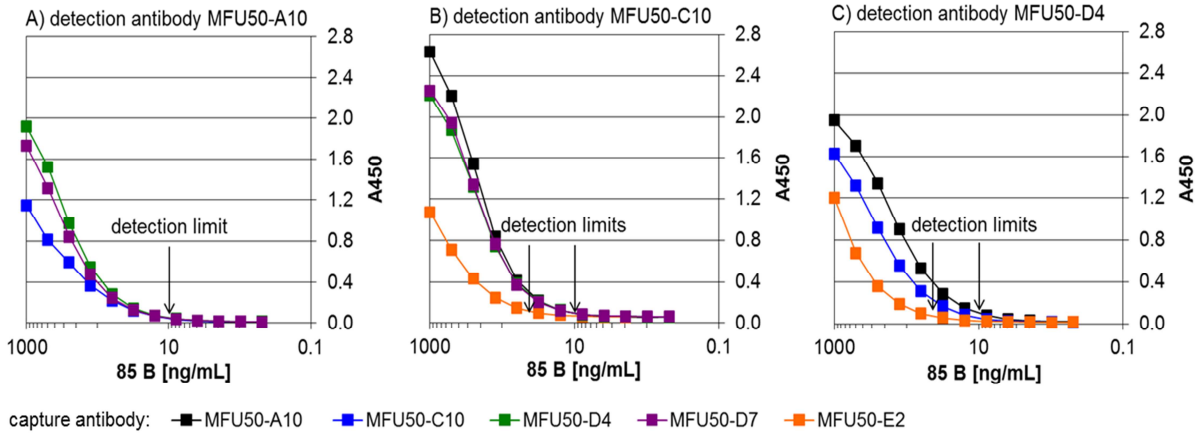


Figure 58: α-85 B sandwich antigen titration ELISA
100 ng of capture scFv-Fc were directly coated to the wells, after blocking different dilutions of antigen were applied and incubated. Detection of bound antigen with: A) MFU50-A10-scFv-Fc-HRP at ~ 0.05 µg/mL, development with TMB. Negative control (BSA) A_{450}=0.02, B) MFU50-C10-scFv-Fc-HRP at ~ 0.05 µg/mL, development with TMB. Negative control (BSA) A_{450}=0.05, C) MFU50-D4-scFv-Fc-HRP at ~ 0.05 µg/mL, development with TMB. Negative control (BSA) A_{450}=0.02.

The antigen detection limit for capturing with MFU50-A10, MFU50-C10, MFU50-D4 or MFU50-D7 was determined to ~ 10 ng mL^{-1}, independent from the detection antibody. Capturing with MFU50-E2 resulted in a detection limit of ~ 25 ng mL^{-1}. The highest signals were obtained by combination of MFU50-A10 as capture antibody and MFU50-C10-HRP as detection antibody-conjugate, hence this combination was considered most suitable. Mtb culture filtrate (Mtb cultivated in 7H9+ADC+Tween for 3 months at 37 °C) was analysed by α-85 B sandwich titration ELISA (Figure 59: A, red curve), resulting in weak binding barely distinguishable from the medium control (Figure 59: A, pink curve). Mtb culture filtrate was further analysed by direct α-85 B ELISA (Figure 59: A, black curve). In this assay no increase of the signal by increase of the sample volume was obtained. Additionally, Mtb cell extract was analysed by α-85 B sandwich titration ELISA (Figure 59: B, red curve), showing no binding distinguishable from the negative control (except for the highest concentration (Figure 59: B, grey curve). Whereupon, in a direct α-85 B ELISA specific binding to Mtb cell extract was observed (Figure 59: B, black curve).

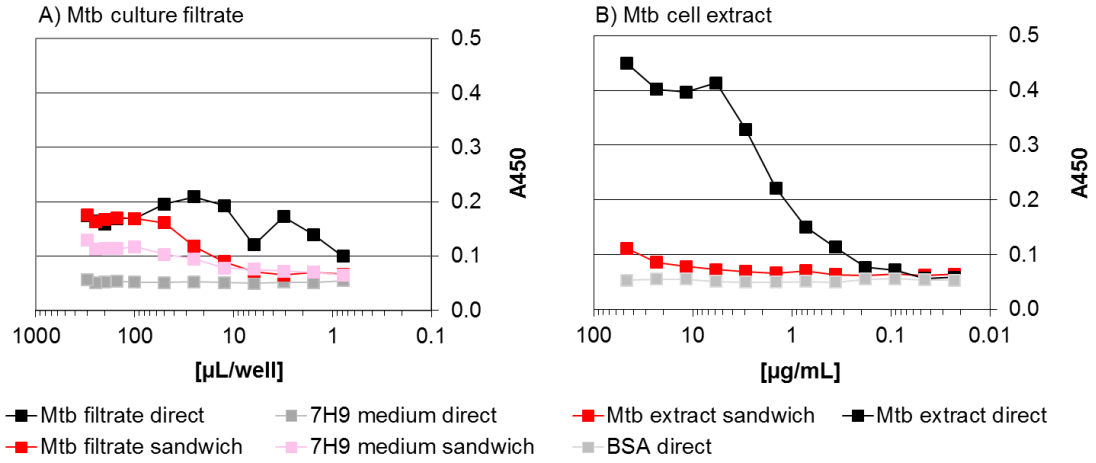


Figure 59: A) Mtb culture filtrate and B) Mtb cell extract titration in α-85 B direct and sandwich ELISA.
Direct ELISA: A) various volumina of Mtb culture filtrate or B) different dilutions of Mtb cell extract were directly coated to the wells, after blocking antigen was detected using MFU50-C10-scFv-Fc-HRP at ~ 0.05 µg/mL, development with TMB.
Sandwich ELISA: 100 ng of capture scFv-Fc MFU50-A10 were directly coated to the wells, after blocking A) various volumina of Mtb culture filtrate or B) different dilutions of Mtb cell extract were applied and incubated, detection of bound antigen with MFU50-C10-scFv-Fc-HRP at ~ 0.05 µg/mL, development with TMB. Negative controls: BSA, *M. smegmatis* cell extract, *M. vaccae* cell extract all A_{450}=0.05.

The failed detection of 85 B in Mtb cells extract in sandwich ELISA compared to the successful detection in direct ELISA was further analysed. It was hypothesized, that sample treatment, especially autoclaving, was the cause for differing antigen detection. Cell extracts were fabricated by autoclaving at 121 °C for 20 min. The influence of sample pre-treatment by heat on 85 B detection was investigated by direct ELISA (Figure 60). Antigen binding was reduced, when 85 B was boiled. Further examination of the susceptibility of 85 B to heat was carried out by analytical SEC (Figure 61).

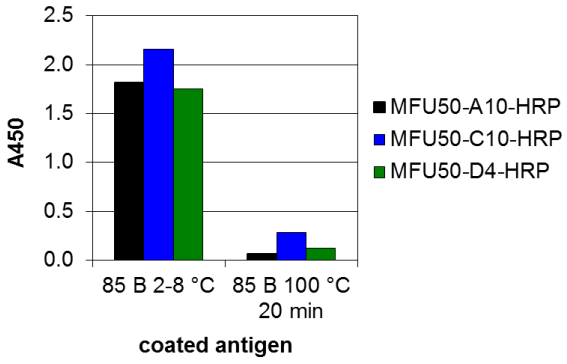


Figure 60: Detection of boiled 85 B by α-85 B direct ELISA.
100 ng of antigens in PBS (boiled for 20 min at 100 °C or directly taken from storage at 2 – 8 °C) were coated to the wells, after blocking bound antigen was detected using MFU50-A10/-C10/-D4-scFv-Fc-HRP at ~ 0.05 µg/mL, development with TMB. Negative control BSA A_{450}=0.02.

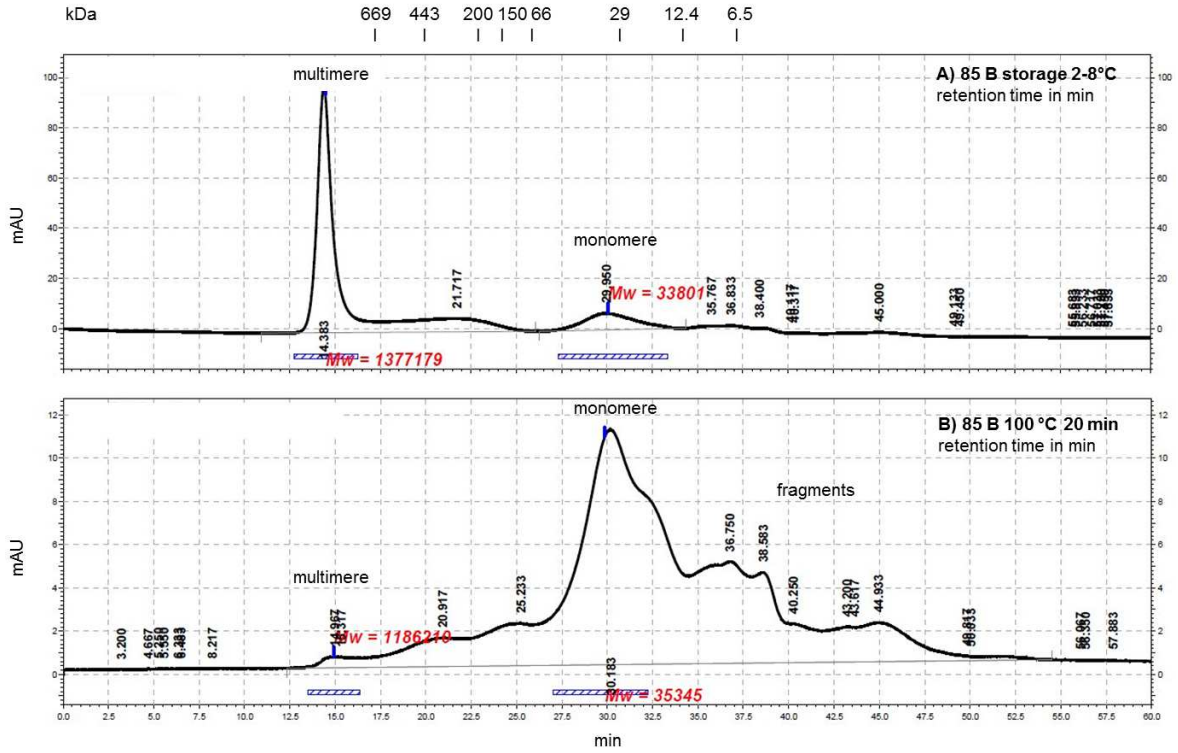


Figure 61: Analytical SEC of antigen 85 B (batch 09-2/1).
A) sample stored at 2 – 8 °C, B) sample boiled for 20 min at 100 °C. 500 µL of sample were applied to Superdex 200 10/300 GL column, running buffer was PBS pH 7.4. Standards: 1. 669 kDa 17 min, 2. 443 kDa 20 min, 3. 200 kDa 23 min, 4. 150 kDa 24 min, 5. 66 kDa 26 min, 6. 29 kDa 31 min, 7. 12.4 kDa 34 min, 8. 6.5 kDa 37 min. Analysis was performed with technical assistance of Wiebke Prilop (Lionex GmbH).

The theoretical molecular mass of an 85 B monomer (recombinant with C-terminal His-tag) was computed with the ExPASy prot param tool to 35.4 kDa. The untreated sample of 85 B displayed a small monomer peak at 33.8 kDa and a dominant multimere peak with a molecular mass out of the measurement range but definitely greater than 669 kDa (Figure 61: A). In comparison to that the boiled 85 B sample offered a dominant peak at the size of a monomer (35.3 kDa), multiple peaks with lower molecular mass and a few non-dominant multimere peaks (Figure 61: B). Interestingly, aggregation was greater in the untreated sample than in the boiled sample. It was concluded that the aggregation was benefitting sandwich detection. If multimers were detected by sandwich ELISA, than use of the same antibody for capturing and detection should be possible. Experimental study by α-85 B sandwich ELISA (Figure 62) verified this assumption. In the untreated as well as in the boiled sample sandwich detection with only one antibody was possible (Figure 62, arrows).

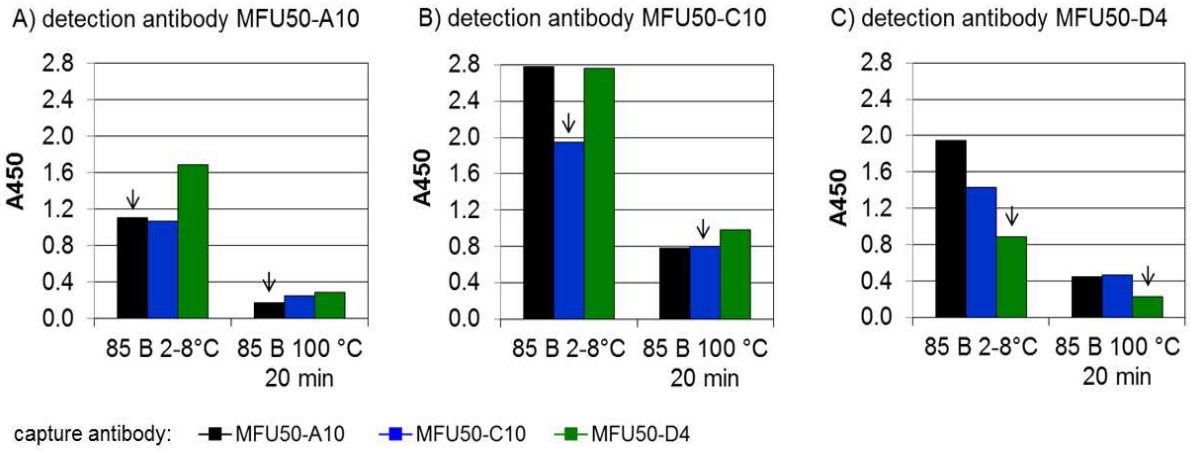


Figure 62: Detection of 85 B multimers in α-85 B sandwich ELISA.
100 ng of capture scFv-Fc were coated to the wells, after blocking 100 ng of 85 B in PBS (untreated or boiled for 20 min at 100 °C) were applied to the wells and incubated, bound antigen was detected using MFU50-A10/-C10/-D4-scFv-Fc-HRP at ~ 0.05 µg/mL, development with TMB. Negative control BSA A_{450}=0.05. Multimer-detection is indicated by arrows.

Further study of the binding properties of the α-85 B scFv-Fc was carried out by SPR. Therefore the human antibody capture kit and a CM5 chip were used. ScFv-Fc antibodies were successfully bound to the α-human IgG antibody on the surface, but when antigen was applied there was either no signal obtained or the signal lay out of the measurement range. It was reasoned that the multimeric structure of the recombinant antigen was responsible for that. Boiling of recombinant 85 B reduced the amount of aggregates to a minimum (compare SEC Figure 61: B), hence the antigen was pretreated this way before SPR. Now an association of antigen and antibody was detectable but no dissociation followed. It was assumed that the debris of boiled antigen 85 B caused disturbing flow characteristics. With this antigen preparation no determination of binding characteristics via SPR was possible

4.2.5.4 A-85 B sandwich LFIA

All α-85 B scFv-Fc were conjugated to colloidal gold (40 nm) by Lionex GmbH. General conjugation-parameters and an overview of the performed experiments is given in Table 48. Every scFv-Fc-gold conjugate reacted with 400 ng 85 B dispensed on a nitrocellulose membrane (direct assay), the weakest signal was obtained by MFU50-E2-gold (Table 48: B) and therefore this conjugate was processed no further. Cross reactivity with different antigens was determined in direct assays, revealing slight cross reactions of MFU50-C10-gold with Rv0211 and MPB70 (Table 48: C), hence this conjugate was processed no further. Sandwich detection of 85 B was carried out with MFU50-A10, MFU50-C10 or MFU50-D4 as capture antibodies and the remaining scFv-Fc-gold conjugates as detection antibodies. Sandwich detection was successful, but

background appeared using MFU50-C10 for capturing (exemplary displayed for MFU50-D4 scFv-Fc-gold in Figure 63: B). Thus capturing was further accomplished with MFU50-A10 and MFU50-D4. Sandwich detection was most sensitive using MFU50-A10 as capture antibody and MFU50-D4-gold as detection antibody (detection limit ≤ 50 ng mL^{-1}, Table 48: E). These results were reproducible in small scale but after upscale of the MFU50-D4-gold conjugation background reactions occurred.

Table 48: Overview α-85 B LFIA development.

			scFv-Fc-gold		
	MFU50-E2	**MFU50-C10**	**MFU50-A10**	**MFU50-D7**	**MFU50-D4**
A) conjugation-parameters					
pH	6.4	9.8	6.8	8.4	8.8
c (scFv-Fc) [µg/mL]	75	100	75	75	75
blocking agent	BSA	BSA	BSA	BSA	BSA
B) direct assay (antigen detection)					
85 B	weak	strong	strong	strong	strong
C) direct assay (cross reactions)					
Rv0467	-	none	none	none	none
Rv3019	-	none	none	none	none
Rv0211	-	weak	none	none	none
Rv1636	-	none	none	none	none
MPB70	-	weak	none	none	none
D) sandwich assay (antigen detection)					
capture scFv-Fc					
MFU50-A10	-	-	-	specific	specific
MFU50-C10	-	-	unspecific	unspecific	unspecific
MFU50-D4	-	-	specific	-	-
MFU50-D7	-	-	-	-	-
MFU50-E2	-	-	-	-	-
E) detection limit [ng mL^{-1}]					
MFU50-A10	-	-	-	≤ 100	≤ 50
MFU50-D4	-	-	≤ 100	-	-
F) reproduction					
MFU50-A10	-	-	-	-	successful
G) upscale					
MFU50-A10	-	-	-	-	unspecific

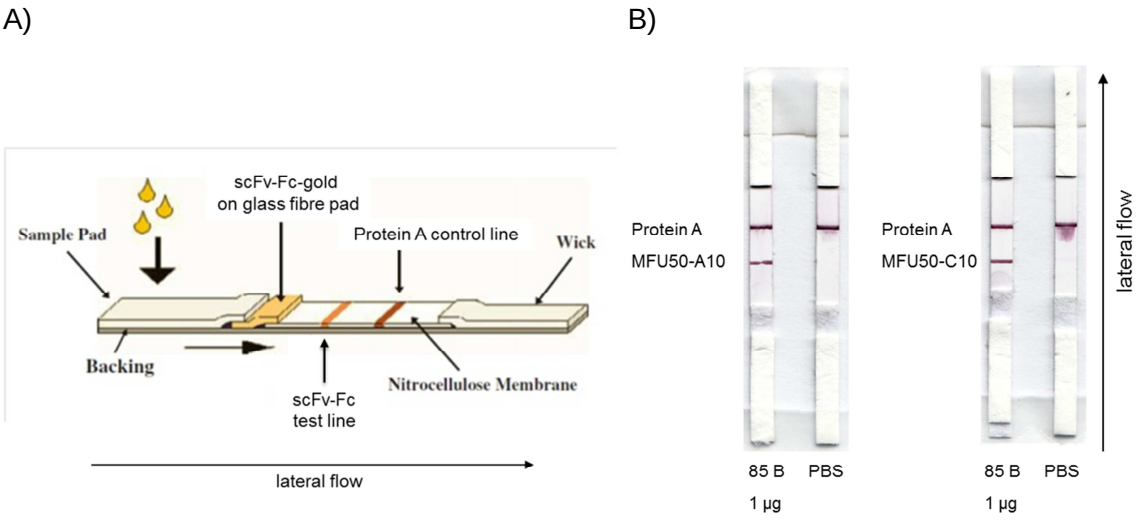


Figure 63: A-85 B sandwich LFIA, configuration and capture antibody selection.
A) general configuration of LFIA, modified after (Wong and Tse, 2009) B) 1 µL cm^{-1} of MFU50-A10 or MFU50-C10 scFv-Fc (each 1 mg mL^{-1}) were dispensed onto nitrocellulose membrane, membrane was cut in 0.4 cm strips, glass fibre pad soaked with MFU50-D4 scFv-Fc-gold was applied, LFIA were assembled. Assay with 120 µL of PBS pH 7.4 as negative control and 120 µL PBS spiked with 1 µg of antigen 85 B as positive control. Scan after 15 min.

Removal of these background reactions was attempted by substituting constituents of the LFIA, dilution of the antibodies, blocking trials and variation of pH. The influence of individual conditions is displayed in Table 49.

Table 49: Influence of conditions in α-85 B sandwich LFIA.

		influence on	
diversified condition		**background**	**antigen recognition**
constituents	buffer batch	none	none
	gold sol batch	none	none
dilutions	scFv-Fc-gold conjugate	weaker with dilution	
	capture scFv-Fc	weaker with dilution	
blocking	membrane	none	none
	glass fibre pad	none	none
	running buffer	none	none
	capture scFv-Fc	none	none
electrochemical	pH	strong	strong

The background reaction was predominantly influenced by pH. LFIA at the pH of scFv-Fc-gold conjugation (pH 8.8 for MFU50-D4) removed the background. Unfortunately antigen detection at pH 8.8 was impossible in a sandwich assay and in a direct assay as well. In summary antigen detection at physiological pH 7.4 was possible (with background) and not possible at conjugation pH 8.8 (no background).

Several experiments were carried out to determine a procedure that would allow sensitive antigen detection and a low background combined with good feasibility (data

not shown). The best option for a manageable LFIA was to add a diluent to the sample before applying it to the sample well. Therefore 1/3 sample volume (50 µL) of conjugation buffer (2.2 mM borax pH 8.8) enriched with 8 % (w/v) BSA were mixed with 100 µL sample and applied to the LFIA. After 15 – 20 min results were evaluated. A detection limit of ≤ 5 ng mL^{-1} was determined (Figure 64).

No reaction with Mtb cell extract or culture filtrate (concentrated and unconcentrated) was observed. Spiking experiments with human sputum and urine samples were performed by Lionex GmbH. The spike was fully retrieved from the urine samples, but only to 50 % from sputum samples. All available sputum samples of confirmed TB patients were autoclaved prior to storage at 2 – 8 °C at Lionex GmbH. Previous experiments with boiled 85 B (chapter 4.2.5.3) showed an extensive drop down of the signals in sandwich ELISA compared to untreated 85 B, hence no further examination of the allocatable samples was carried out.

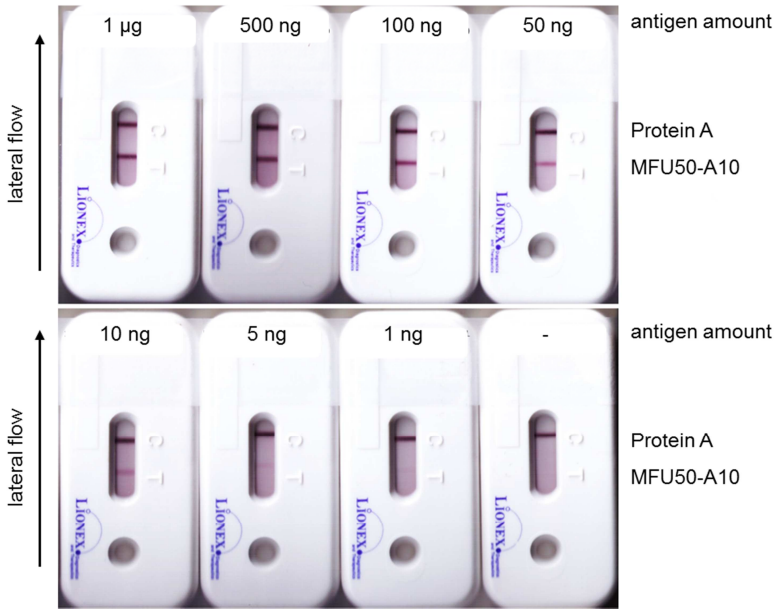


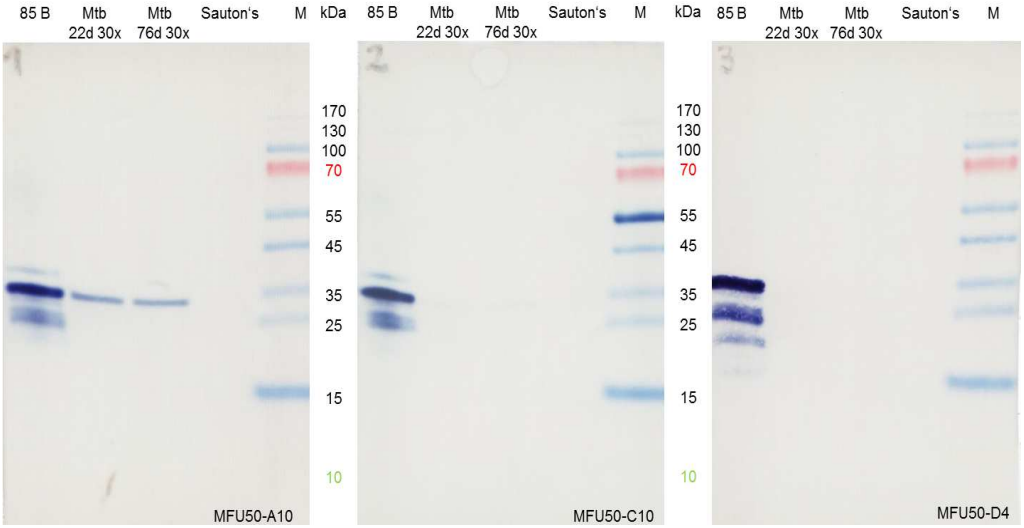
Figure 64: A-85 B sandwich LFIA, determination of detection limit.
MFU50-D4 scFv-Fc-gold on glass fibre pad for detection, MFU50-A10 scFv-Fc on membrane for capturing (lower line), Protein A as control (upper line). Measurement of a dilution series of 7H9 medium spiked with 85 B. 100 µL sample + 50 µL 2.2 mM borax pH 8.8 containing 8 % BSA were applied on sample pad, read out after 20 min.

4.2.5.5 A-85 B immunoblot

Sandwich detection of antigen 85 B in Mtb culture filtrates and cell extracts was complex in previously described sandwich ELISA and LFIA. As an alternative a direct α-85 B immunoblot assay was developed to avoid the challenge of sample pre-treatment in sandwich detection. Mtb culture filtrates may contain a too small amount of antigen to be detectable in the described assays. Therefore concentration of culture filtrates was

carried out. To avoid enrichment of protein ingredients from nutrient broth, Mtb was cultured in Sauton's minimal medium. An older culture (54 days old at the start of the experiments) and a fresh inoculated culture were examined. Weekly samples were taken, concentrated 20 to 30 fold and analysed by reducing gel analysis via Tape Station and α-85 B immunoblot. No 85 B was detected in 7 and 16 days old cultures by either means (data not shown). After 22 days a protein band at ~30 kDa (according to Tape Station analysis, Figure 65: B) was recognized by MFU50-A10 in immunoblot (Figure 65: A). Additionally, 85 B expression was verified for 61 and 70 days old cultures (data not shown). The Tape Station and α-85 B immunoblot results for 22 and 76 days old cultures are given in Figure 65.

A)



B)

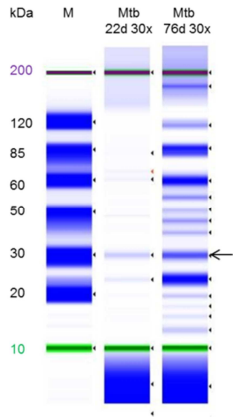


Figure 65: A) α-85 B immunoblot and B) Tape Station analysis of concentrated Mtb culture filtrates (22 d or 76 d at 37 °C)
A) 100 ng of recombinant 85 B, 25 µL of concentrated Mtb culture filtrates (after 22 or 76 days at 37 °C in Sauton's medium) and 8 µL of marker (M) were separated on reducing 12 % SDS-PAGE and electro blotted to a PVDF membrane. After blocking, blots were incubated with scFv-Fc-HRP, development with TMB. B) Samples were directly analysed by reducing gel analysis via Tape Station.

In summary antigen 85 B was detectable in concentrated culture filtrates of Mtb after 17 – 22 days of cultivation in Sauton's medium at 37 °C via α-85 B immunblot. A more accurate determination of the necessary cultivation time was not possible due to the lack of access to the L3 facility in Hannover. The positively tested culture filtrates were further analysed by α-85 B direct and sandwich ELISA in addition to α-85 B sandwich LFIA. There was no reaction detectable via LFIA. In ELISA a massive background reaction of the culture filtrates with TMB was observed.

4.3 Summary

For clarity the properties of the generated antibodies are summarized in Table 50, Table 51 and Table 52.

Table 50: Properties of generated antibodies part 1.

	no.	1	2	3	4	5	6	7	8
	antigen	LAM	16 kDa	16 kDa	16 kDa	16 kDa	16 kDa	16 kDa	16 kDa
	antibody	Lx143	SH365-C8	SH365-E4	SH365-H4	SH451-C11	SH451-F11	L16-3-D12	L16-3-E12
	origin	hybridoma	HAL7	HAL7	HAL7	HAL7	HAL7	HAL7	HAL7
germline gene	heavy chain	VH6	VH3	VH3	VH3	VH3	VH3	VH3	VH3
	light chain	VK1	VL1	VL1	VL1	VL1	VL1	VL1	VL1
antigen binding	scFv	not verified	verified	verified	verified	verified	verified	verified	verified
	scFab	not verified	-	-	-	-	-	-	-
	scFv-Fc	not verified	-	-	-	-	-	-	verified
	scFab-Fc	verified	-	-	-	-	-	-	-
purification in E. coli	scFv	0 mg/L	46 mg/L	34 mg/L	5 mg/L	2 mg/L	2 mg/L	36 mg/L	16 mg/L
	scFab	0 mg/L	-	-	-	-	-	-	-
purification in HEK293-6E	scFv-Fc	0 mg/L	-	-	-	-	-	-	112 mg/L
	scFab-Fc	24 mg/L	-	-	-	-	-	-	-
epitope		-	partial continuous	partial continuous	partial continuous	partial continuous	partial continuous	partial continuous	partial continuous
affinity		-	7.44E-08 M	2.08E-07 M	1.13E-07 M	1.27E-07 M	1.13E-07 M	2.76E-07 M	1.06E-08 M
indirect ELISA (scFv)	antibody titration	-	okay	okay	okay	okay	okay	okay	okay
indirect ELISA (Fc-fusion)	antibody titration	okay	-	-	-	-	-	-	okay
	antigen titration	-	-	-	-	-	-	-	yes
	detection limit	-	-	-	-	-	-	-	10 ng/mL
	Mtb extract	yes	-	-	-	-	-	-	yes
	Mtb filtrate	yes	-	-	-	-	-	-	yes
	BCG extract	yes	-	-	-	-	-	-	no
	BCG filtrate	no	-	-	-	-	-	-	no
indirect immunoblot	antigen	-	yes	yes	yes	yes	yes	yes	yes
cross reactions	Mtb antigens	-	none	none	none	none	none	85 A, 85 B	85 A, 85 B
conjugation to HRP		-	-	-	-	-	-	-	yes
sandwich ELISA	antigen titration	-	-	-	-	-	-	-	no reaction
	detection limit	-	-	-	-	-	-	-	-
	Mtb extract	-	-	-	-	-	-	-	-
	Mtb filtrate	-	-	-	-	-	-	-	no reaction

Table 51: Properties of generated antibodies part 2.

	no.	9	10	11	12	13	14	15	16	17	18
	antigen	CFP-10	CFP-10	CFP-10	85 D	85 D	85 D	85 A	85 A	85 A	85 A
	antibody	MFU48-C8	MFU48-E1	MFU48-G4	MFU51-A6	MFU51-B10	MFU51-C2	MFU12-D8	MFU53-A3	MFU53-F3	MFU53-G2
	origin	HAL7	HAL7	HAL7	HAL7	HAL7	HAL7	HAL7	HAL7	HAL7	HAL7
germline gene	heavy chain	VH6	VH3	VH6	VH1	VH3	VH3	VH3	VH3	VH1	VH3
	light chain	VL3	VL1	VL3	VL3	VL1	VL2	VL6	VL2	VL3	VL1
antigen binding	scFv	verified	verified	verified	verified	verified	verified	verified	verified	verified	verified
	scFv-Fc	verified	verified	verified	verified	verified	verified	verified	verified	verified	verified
purification in HEK293-6E	scFv-Fc	111 mg/L	158 mg/L	174 mg/L	73 mg/L	194 mg/L	53 mg/L	37 mg/L	176 mg/L	51 mg/L	215 mg/L
epitope		continuous	discontinuous?	continuous	continuous	continuous	continuous	continuous	continuous	discontinuous?	continuous
indirect ELISA (Fc-fusion)	antibody titration	okay	okay	okay	okay	okay	okay	okay	okay	unspecific	okay
	antigen titration	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
	detection limit	300 ng/mL	300 ng/mL	300 ng/mL	-	-	-	10 ng/mL	10 ng/mL	60 ng/mL	60 ng/mL
	Mtb extract	no	no	no	yes	no	no	no	no	no	no
	Mtb filtrate	no	no	no	yes	yes	no	no	no	no	no
	BCG extract	no	no	no	yes	no	no	no	no	no	no
	BCG filtrate	no	no	no	no	no	no	no	no	no	no
indirect immunoblot	antigen	not operable	not operable	not operable	yes	yes	yes	yes	yes	yes	yes
cross reactions	Mtb antigens	none	none	none	85 A, 85 B	none	85 A, 85 B	none	85 D	85 B	85 B
conjugation to HRP		yes	yes	yes	-	-	-	-	yes	-	yes
sandwich ELISA	antigen titration	no	no	no	-	-	-	yes	yes	yes	yes
	detection limit	-	-	-	-	-	-	125 ng/mL			
	Mtb extract	-	-	-	-	-	-	no			
	multimer recognition	-	-	-	-	-	-	yes			
conjugation to colloidal gold		yes	yes	yes	-	-	-	yes	yes	-	yes
direct LFIA	antigen	yes	yes	yes	-	-	-	yes	yes	-	yes
sandwich LFIA	antigen titration		unspecific		-	-	-	unspecific			

Table 52: Properties of generated antibodies part 3.

	no.	19	20	21	22	23
	antigen	85 B	85 B	85 B	85 B	85 B
	antibody	MFU50-A10	MFU50-C10	MFU50-D4	MFU50-D7	MFU50-E2
	origin	HAL7	HAL8	HAL7	HAL7	HAL7
germline gene	heavy chain	VH3	VH3	VH3	VH3	VH3
	light chain	VL8	VK1	VL2	VL3	VL7
antigen binding	scFv	verified	verified	verified	verified	verified
	scFv-Fc	verified	verified	verified	verified	verified
purification in HEK293-6E	scFv-Fc	198 mg/L	197 mg/L	194 mg/L	183 mg/L	50 mg/L
epitope		continuous	continuous	continuous	continuous	continuous
affinity		not operable	not operable	not operable	not operable	not operable
indirect ELISA	antibody titration	okay	okay	okay	okay	okay
	antigen titration	yes	yes	yes	yes	yes
	detection limit	10 ng/mL	5 ng/mL	30 ng/mL	30 ng/mL	10 ng/mL
	Mtb extract	yes	no	no	no	no
	Mtb filtrate	yes	no	no	no	no
	BCG extract	yes	no	no	no	no
	BCG filtrate	yes	no	no	no	no
indirect immunoblot	antigen	yes	yes	yes	yes	yes
cross reactions	Mtb antigens	85 A, 85 D	none	85 A	none	none
conjugation to HRP		yes	yes	yes	-	-
sandwich ELISA	antigen titration	yes	yes	yes	yes	yes
	detection limit			10 ng/mL		
	Mtb extract			no		
	Mtb filtrate			no		
	conc. Mtb filtrate			unspecific		
	multimer recognition			yes		
direct ELISA	Mtb extract			yes		
	Mtb filtrate			unspecific		
direct immunoblot	conc. Mtb filtrate			yes		
conjugation to colloidal gold		yes	yes	yes	yes	yes
direct LFIA	antigen	yes	yes	yes	yes	yes
sandwich LFIA	antigen titration	yes	unspecific	yes	yes	yes
	detection limit			5 ng/mL		
	Mtb extract			no		
	Mtb filtrate			no		
	conc. Mtb filtrate			no		

5 Discussion

For the detection of Mtb, antibodies against the antigens 16 kDa, CFP-10, 85 A, 85 B and 85 D were isolated by panning of the naïve human phage display libraries HAL7/8. In addition, a recombinant α-LAM antibody was generated from an IgM expressing hybridoma clone. Furthermore, these antibodies were purified in different formats, biochemically characterised and examined regarding their suitability in different Mtb antigen detection assays.

5.1 LAM

The human naïve phage display libraries HAL7/8 (Hust *et al.*, 2011) were screened by panning on immobilized LAM. Unfortunately, no LAM specific antibodies could be isolated. LAM is a lipopolysaccharide and appears to be a poor immunogen. Carbohydrates in general show only inadequate immunogenicity (Hirvonen *et al.*, 2013). If polyclonal or monoclonal α-carbohydrate antibodies can be raised by immunization, they are typically of the IgM class (Kannagi and Hakomori, 2001). It seems efficient binding of carbohydrate-specific antibodies depends on multivalency (Karush 1970; Winter et al. 1994). In this work a monovalent phage display technique with naïve, IgM originated scFv libraries was used. Therefore a multivalent display technique, by i.e. shortening of the peptide linker and following multimerization of scFv due to steric hindrance (Ravn *et al.*, 2004), may have been more successful. In addition, the presentation of the antigen on the surface of microtiter plates can be suboptimal. This could be solved by panning on LAM-carrier protein conjugates or panning on magnetic beads.

Because no scFv were selected, further work was pursued with the IgM expressing hybridome Lx143. From this clone several slightly different variable gene segments were isolated, but none was coding for the functional antibody. Isolation of a great variety of antibody genes from one cell line, ranging from slight differences in one allel to different light or heavy chain types, was reported by several authors before (Chen *et al.*, 1986; Shimizu *et al.*, 1991; Pauza *et al.*, 1993). Aberrant variable gene transcripts may derive either from the non-secreting myeloma fusion partner or from unproductively rearranged alleles present in the B cell (Irani *et al.*, 2008). Furthermore, hybridomas grow uncoupled from any regulatory apparatus allowing for allelic dysregulation, and several passages of the cells increase the risk of mutation leading to heterologous culture (Toleikis and Frenzel, 2012). In addition, the amplification process itself can introduce variations, because unknown sequences have to be amplified by use of wobbled oligonucleotides

and hybridization conditions allowing for mismatches (Dübel *et al.*, 1994). Functional scFv can be chosen from a variety of possible V_H/V_L combinations by phage display and subsequent panning on antigen (Krebber *et al.*, 1997). In this study the functional protein sequence has been already known and corresponding oligonucleotides were constructed to alter the isolated DNA sequences.

5.1.1 A-LAM scFv/scFab

A scFv construct of the original IgM was generated. The expression level of soluble scFv was low and no antigen recognition in ELISA was detectable, further antibody purification was not possible. Production levels in *E. coli* for scFv derived from hybridoma cell lines can vary heavily. On the one hand intrinsic features of the encoding genes (i.e. codon usage) can influence the expression (Tiwari *et al.*, 2010). On the other hand the folding efficacy is crucial, because not correctly folded antibodies cannot bind antigen or accumulate into insoluble inclusion bodies (Li *et al.*, 1999; Zhu *et al.*, 2013). Codon optimization and co-expression with molecular chaperones can result in higher amounts of soluble scFv (Min *et al.*, 2010). The reducing chemical environment of the cytoplasm in *E. coli* inhibits the formation of intradomain disulfide bridges necessary for the correct folding of scFv (Skerra, Plückthun 1988). In this study scFv were fused with the N-terminal leader peptide pelB that directs the recombinant protein into the periplasm of *E. coli*, where it can be properly folded in an oxidizing environment (Popplewell et al. 2005). The absence of constant regions in scFv, in comparison to the parental IgM, may lead to conformational changes of the paratope or reduced stability. Further, glycosylation may contribute to antigen binding and increase solubility and stability, which cannot be achieved by expression in *E. coli* (Lizak *et al.*, 2011). In this particular case, the antigen is a lipopolysaccharide, indicating a potential role of glycosylation in antigen recognition. Finally, the scFv may have a too low apparent affinity to create a detectable signal in the performed ELISA. IgM antibodies are produced early during immune responses, before somatic hypermutation and affinity maturation have taken place (Murphy *et al.*, 2008), and usually recognize repetitive epitopes like polysaccharides on bacterial cell walls (like LAM). The avidity effect of the ten-arm IgM can neutralize low affinities, but is missing in the monovalent scFv (Roggenbuck *et al.*, 1994).

In order to stabilize the antibody, a format change from scFv to scFab (with mouse C_L and C_H1) was performed. The results were very similar to those determined for the scFv. The expression of soluble antibody was low, no reaction in ELISA, and no purification possible. A positive effect of the constant regions in the scFab construct could not be detected.

5.1.2 A-LAM scFv-Fc/scFab-Fc

To fetch an avidity effect and facilitate glycosylation, the scFv was fused to a human IgG1 Fc-part in an eukaryotic expression system (HEK293-6E cells, (Jäger *et al.*, 2013)). There was no detectable expression of scFv-Fc and hence no purification possible.

Finally, the scFab was fused to a human IgG1 Fc-part resulting in a scFab-Fc (scIgG) construct. Interestingly, expression and purification of this antibody was possible. This finding indicates a stabilizing effect of the C_L and C_H1 domains that are present in the scFab-Fc but absent in the scFv-Fc. Furthermore, the avidity effect caused by the Fc-mediated homodimerisation of scFab-Fc (Jäger *et al.*, 2013; Powers *et al.*, 2001) may be responsible for an increased apparent affinity. Moreover, a possible contribution of glycosylation to antigen binding cannot be excluded. The antibody yield was low (24 mg/L) in comparison to scFv-Fc antibodies (36 – 215 mg/L) produced in this study, probably due to the increased size of a scFab-Fc or due to the hybridoma origin.

LAM is a component of the mycobacterial cell wall, and it's presence is not limited to virulent strains of mycobacteria (Besra and Brennan, 1997; Chatterjee and Khoo, 1998). A reaction of the α-LAM scFab-Fc with Mtb and BCG cell extracts and purified Mtb/BCG LAM was detected by indirect ELISA. But the reaction with BCG derived LAM was weaker in both cases, indicating potential differences between Mtb LAM and BCG LAM. Similar results were encountered for the parental IgM (personal communication with F. Jonas) and for other monoclonal antibodies raised against Mtb LAM (Prinzis *et al.*, 1993).

In future, purification of the scFab, eventually by refolding inclusion bodies from *E. coli* (Min *et al.*, 2010) or by papain digestion of the scFab-Fc, could enlighten the role of avidity and glycosylation in antigen binding. If any scFab bound LAM, then the avidity effect would not be necessary for antigen recognition. If an *E. coli* derived scFab bound LAM, then glycosylation would not be essential for antigen binding.

5.2 16 kDa

5.2.1 Selection of antibodies against 16 kDa

Seven individual α-16 kDa antibodies were isolated from the human naïve libraries HAL4/7. Interestingly, all these antibodies share the same variable gene segment of the heavy chain. In addition, respectively six out of seven antibodies have the same joining gene segment of the light chain, the same variable gene segment of the light chain or the

same joining gene segment of the heavy chain. The highest variability is found in the diversity gene segment of the heavy chain. Antigen binding is mainly influenced by CDR3 length and sequence variation (Davis *et al.*, 1997). Especially the diversity of CDR3 of the heavy chain is often enough to mediate antigen specificity, and somatic mutations only in V_H CDR3 can increase the affinity (Xu, Davis 2000). According to IMGT (Lefranc *et al.*, 1999) there are 7 subfamilies of human V_H (mathematical probability of IGHV3 = 14.3 %), 53 genes of V_H3 (mathematical probability of IGHV3-15 = 1.9 %) and 8 allels of gene IGHV3-15 (mathematical probability of IGHV3-15*01 = 12.5 %). In summary, the mathematical probability for the IGHV3-15*01 gene is about 0.03 %. Further, the mathematical probability for a combination of IGHV3-15*01 with IGLV1-47 and IGLJ3, which are present in every α-16 kDa clone, is about 0.00006 %. Interestingly, this combination was selected from two different libraries. The minor chances for a combination of these genes, in addition to the appearance in two libraries, suggest a preferential combination of these gene segments in phage display libraries and/or *in vivo*. Indeed, the variable gene segments V_H3 and $V_λ1$ are overrepresented *in vivo* (Knappik *et al.*, 2000) and in naïve phage display libraries (Schofield *et al.*, 2007). Furthermore, scFv antibodies with a combination of V_H3 and $V_λ1$ are the most abundant in the HAL7 library with a probability for selection of about 23.3 % (Hust *et al.*, 2011). In phage display, antibodies with higher stability and production yields in *E. coli* are favoured (Loset *et al.*, 2005). Ewert and colleagues found V_H3 to possess the highest thermodynamic stability of all isolated V_H domains, and scFv including V_H3 were the most stable with the highest production yields in *E. coli* (Ewert *et al.*, 2003). Thereby, the combination of V_H3 with $V_λ1$ seems common. Nevertheless, the homogeneity of the α-16 kDa antibodies in combination of not only gene subfamilies but in addition particular genes and allells is remarkable. In this study, none of the selected antibodies against different antigens shared the exact gene combination. It seems there is a preferential combination of genes against the 16 kDa antigen.

5.2.2 Characterization of α-16 kDa antibodies

All scFv were expressed in and purified from *E. coli*, volumetric yields ranging from 2 mg/L (SH451-F11) to 46 mg/L (SH365-C8). ScFv expression in *E. coli* is various, depending on the production system and the individual antibody sequence (Frenzel *et al.*, 2013). An AbCheck analysis (Martin, 1996) revealed an extraordinary methionine in the light chain of SH451-F11, on the contrary the AbCheck analysis of SH365-C8 showed no unusual sequence features. In addition, the quaternary structures of the scFv in PBS pH 7.4 were analysed. The analytical SEC of SH451-F11 offered about 16 % possible multimers, whereupon SH365-C8 showed no aggregates. The predicted grand

average of hydropathicity of SH451-F11 is double as positive as that of SH365-C8, determined by ExPASy Protparam (Artimo *et al.*, 2012). Transient opening of the interface between V_H and V_L domains, allowed by the peptide linker, exposes hydrophobic patches that favour aggregation (Wörn and Plückthun, 2001). Therefore the formation of associates promoted by hydrophobic interactions is more likely for SH451-F11, which is supported by the experimental findings. Although the combination of V_H3 with $V_\lambda1$ was found to produce the most stable scFv, antibody stability and production yields highly depend on the light chain CDR3 sequences (Ewert *et al.*, 2003), that vary by substitution of 1 – 2 amino acids in the α-16 kDa antibodies.

Epitope mapping of the α-16 kDa scFv suggested the recognition of the same epitope "KDFDGRS" by all antibodies. But the reactions with the peptides (and with reduced antigen in immunoblot) were weak, indicating that the epitopes may at least partially be conformational. Cross reactions of L16-3-D12 and L13-3-E12 with 85 A and 85 B were determined by indirect ELISA and direct immunblot, although the sequence "KDFDGRS" is not present in any of the 85 complex antigens (BLASTP, NCBI). Further none of the other α-16 kDa antibodies showed cross reactivity. Suggesting, there are different epitopes of the antibodies not distinguishable by screening overlapping peptide libraries. The complete amino acid sequence "KDFDGRS" is present in three other mycobacteria (*M. canettii*, *M. fortuitum* and *M. avium*) and a few non-pathogenic bacteria (*Neisseria cinerea*, *Saccharomonospora ssp.*, *Jostella marina*), but absent in *M. bovis* identified by BLASTP (NCBI). Thereby, cross reactions with a few non-tuberculous organisms are likely but insignificant, because clinical pictures vary from that of TB. Further, the epitope region "KDFDGRS" shows great immunogenicity. Synthetic peptides including this sequence were found to induce T cell responses in mice (Vordermeier *et al.*, 1993) and humans (Friscia *et al.*, 1995). Furthermore, the epitopes of mouse monoclonal antibodies, human sera and llama VHH antibodies against 16 kDa were found to include "KDFDGRS" (Verbon *et al.*, 1992; Srivastava *et al.*, 2013).

The affinities of the α-16 kDa scFv were determined by SPR, ranging from $2.76x10^{-07}$ M (L16-3-D12) to $1.06x10^{-08}$ M (L16-3-E12). Trilling and colleagues (Trilling *et al.*, 2011) reported α-16 kDa llama VHH antibodies, selected from an immune phage display library, with affinities of maximum $4x10^{-10}$ M. A human α-16 kDa IgA, isolated from a naïve phage display library, had an affinity of 6.99×10^{-8} M (Balu *et al.*, 2011).

Since all antibodies were reactive with one sequence of the antigen, work was pursued with the antibody possessing the highest affinity (L16-3-E12). A slight reaction of this antibody with Mtb cell extracts and culture filtrate was measured by indirect ELISA. The

16 kDa antigen is the most abundant protein in *M. tuberculosis* during its dormant, non-replicative phase but is not present under conditions of logarithmic growth (Yuan et al. 1996; Hu, Coates 1999). Since the cultures examined in this study are usually proliferating, only marginal antigen expression can be expected. In addition, 16 kDa is associated with the mycobacterial cell wall and not secreted (Lee *et al.*, 1992), therefore presence in Mtb culture filtrates can only be expected by cell lysis.

The dodecameric structure of native 16 kDa, probably build up by association of six homodimers (Kennaway, 2005; Srivastava *et al.*, 2013) could facilitate sandwich detection of the antigen with only one antibody. In this work, an α-16 kDa sandwich ELISA with L16-3-E12 scFv-Fc as capture antibody and detection HRP-conjugate was not successful neither with partly multimeric recombinant antigen nor Mtb filtrate. This might be due to sterical inhibition by an epitope blocking arrangement in the protein aggregate, or by a multimer amount below the detection limit.

In future, scFv suitable for 16 kDa sandwich detection may be isolated by modified panning on pre-bound antigen. Therefore native antigen (isolated from Mtb) should be captured by L16-3-E12, and panning on this antigen-antibody complex, with human IgG pre-blocked phages, should be performed. In addition, the performance (affinity, stability, etc.) of these recombinant antibodies can be altered by construction of randomly mutagenized secondary phage display libraries and panning with increased selective pressure (Thie *et al.*, 2009; Steidl *et al.*, 2008). Furthermore, a passive immunization trial with the presented α-16 kDa antibodies is promising. A human α-16 kDa IgA antibody selected by phage display, with an undistinguished conformational epitope, mediated protection against TB in mice transgenic for human CD89 (Balu *et al.*, 2011). Considering the fully human nature of the isolated antibodies and the availability of humanized FcRN mouse models (Proetzel and Roopenian, 2013), a similar approach is possible.

5.3 CFP-10

Three individual scFv antibodies binding CFP-10 were isolated from HAL7/8. The antibodies were purified as scFv-Fc fusions and further characterised. The epitope of MFU48-E1 was not determinable by screening overlapping peptide libraries. Considering that MFU48-E1 was not cross reactive with other Mtb antigens, BSA or with BCG, it was concluded that this antibody recognized a discontinuous epitope. MFU48-C8 and MFU48-G4 were recognizing the same continuous epitope “ERISGDLKT”. Interestingly,

these two antibodies have (except for one amino acid exchange in V_H CDR2) exactly the same heavy chain but much more different light chains. This finding further underlines the key role of the heavy chain in antigen specificity, as mentioned before for the α-16 kDa antibodies and found by other authors (Barbas, 3rd *et al.*, 1993; Xu and Davis, 2000). The combination of MFU48-E1 as capture antibody and MFU48-C8 as detection antibody in sandwich immune assays only yielded unreproducible results. It is possible that MFU48-E1 recognizes an epitope close to or overlapping "ERISGDLKT". Thereby sandwich detection would be sterically inhibited. Furthermore, presuming different epitopes, the small size of the antigen (~ 10 kDa) and the width of a scFv arm (~ 4 nm (Boehm *et al.*, 1999)) indicate sterical inhibition by the capture antibody. Moreover, considering that MFU48-E1 is presumed to recognize a discontinuous epitope, it is possible that binding of one antibody to "ERISGDLKT" causes a change in antigen conformation and therewith disables the binding of MFU48-E1. To investigate the reaction with "native" antigen, the α-CFP-10 antibodies were probed with Mtb cell extract and culture filtrate. There was no measurable binding in ELISA. Since CFP-10 is produced early during cultivation and the examined cultures are mostly a few weeks old, there was enough time for degradation. Further, secreted CFP-10 forms a tight 1:1 complex with ESAT-6 (Figure 66: A) (Renshaw *et al.*, 2002). Once bound by an α-CFP-10 antibody, the rest of the CFP-10 site of the complex is probably blocked by ESAT-6 (Figure 66: B).

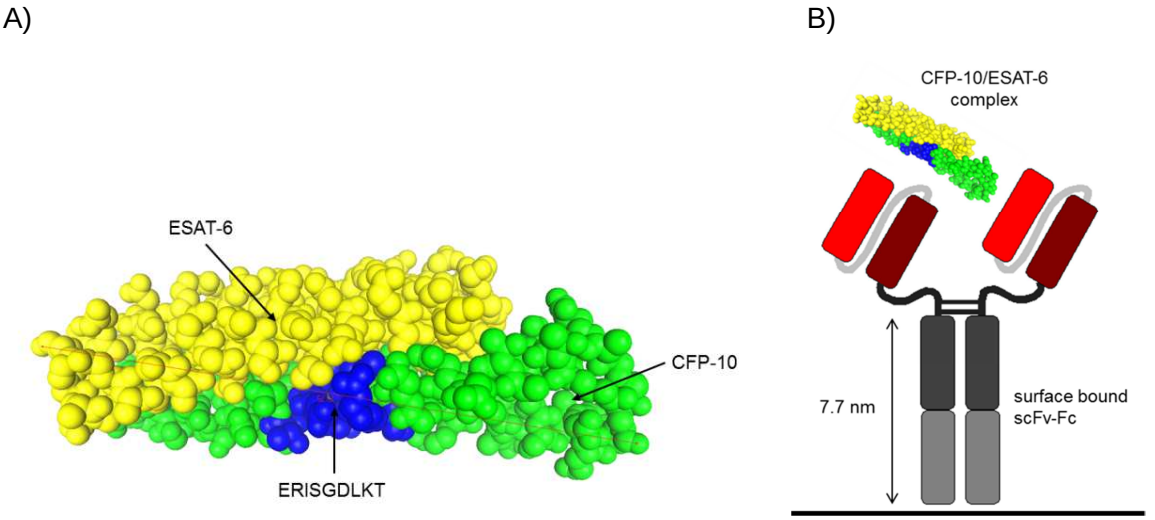


Figure 66: A) 3D structure of the CFP-10/ESAT-6 complex, epitope of α-CFP-10 antibodies, B) possible sterical inhibition of CFP-10 sandwich binding by ESAT-6.
Pdb3fav (Resolution 2.15 Å, (Poulsen *et al.*, 2009)) was modified with 3D molecule viewer (Invitrogen). Only protein chains C (CFP-10, green) and D (ESAT-6, yellow) are shown, atoms are displayed as space filling balls, the epitope "ERISGDLKT" is marked in blue. Antibody symbol adopted from M. Hust with permission.

Thus it seems more promising to target the CFP-10/ESAT-6 complex and not individual antigens. Since α-CFP-10 antibodies were already isolated, further work should focuss on the isolation of α-ESAT-6 antibodies. In this work, no antibodies against ESAT-6 could be selected from HAL7/8. But this finding does not mean that there are no ESAT-6 binders in those libraries. ESAT-6 is a small protein (6 kDa). Thereby antigen presentation on the surface of a MTP can be suboptimal. A different approach by fusion of ESAT-6 to a carrier protein, biotinylation or by panning on magnetic beads could improve antigen presentation. Furthermore, to avoid the selection of sterically inhibited antibodies (in sandwich detection), panning on pre-bound CFP-10/ESAT-6 heterodimer could be useful. Therefore, the complex must be isolated from Mtb culture filtrate or by *in vitro* fusion of the antigens. The α-CFP-10 antibodies could be used for capturing, and α-ESAT-6 antibodies may be isolated by panning with human IgG pre-blocked HAL7/8 phages.

5.4 85 B

5.4.1 Selection of antibodies against 85 B

Five individual antibodies were selected from the human naïve libraries HAL7/8 (Hust *et al.*, 2011). The examined antibodies showed homogeneity in the subfamily of the variable gene segment of the heavy chain (all V_H3). In this study 18 out of 22 antibodies against different epitopes have a V_H3 domain, indicating this variable gene segment of the heavy chain is advantageous. This finding correlates with the overrepresentation of V_H1 and V_H3 in the HAL7/8 libraries (Frenzel *et al.*, 2012) and *in vivo* (Knappik *et al.*, 2000). Further, V_H3 was found to possess the highest thermodynamic stability of all isolated V_H domains, and scFv including V_H3 were the most stable with the highest production yields in *E. coli* (Ewert *et al.*, 2003). Four α-85 B antibodies have a lambda light chain, one a kappa light chain. In this study only one out of 22 clones has a kappa light chain, suggesting an advantage of lambda scFv in the selection process. Interestingly, in humans kappa antibodies outnumber lambda antibodies 1:20 (Murphy *et al.*, 2008). Therefore the advantage of lambda antibodies seems to be limited to *E. coli* associated applications. It was found that lambda scFv are expressed in higher yields in *E. coli* compared to kappa scFv, which can be beneficial in phage display (Frenzel *et al.*, 2012; Hust *et al.*, 2011; Loset *et al.*, 2005). The antibody MFU50-C10 has a germline combination of V_H3 and $V_κ1$, which is common *in vivo* and in naïve libraries (Hust *et al.*, 2011; Schofield *et al.*, 2007; Knappik *et al.*, 2000). The lambda germline sequences present in the other antibodies are from the subfamilies 1, 2, 3, 7 and 8. Subfamilies

Vλ1,2 and 3 are dominant *in vivo*, whereupon Vλ7 and 8 are rare *in vivo* (Knappik *et al.*, 2000; Schofield *et al.*, 2007). In the HAL7 library Vλ7 and 8 are more frequent (Hust *et al.*, 2011). Thereby, phage display allows selection of scFv (i.e. with Vλ7) that can hardly be raised by immunization techniques, probably exhibiting characteristics rarely found *in vivo*.

5.4.2 Characterization of α-85 B antibodies

All α-85 B antibodies recognized continuous sequences of the antigen, and the corresponding epitopes were determined by screening overlapping peptides immobilized on a cellulose membrane. MFU50-D4 recognized “AFSRPGLPVEYL” and MFU50-D7 recognized “AFSRPGLPV”. This epitope region seems to be a potent T cell antigen. Synthetic peptides including this sequence were found to induce cytokine release or immune cell proliferation in peripheral blood of individuals with varying TB status (Huygen et al. 1994; Launois et al. 1994; Roche et al. 1994; Commandeur et al. 2011). Yet only weak human B cell responses against these peptides were reported (Roche et al. 1994; Shen et al. 2009). Therefore generation of antibodies against this region by immunization could be difficult. In this work, human antibodies were successfully generated by screening phage display libraries, fortifying the advantage of phage display technology over conventional immunization methods. Protein sequence comparison of the 85 complex proteins revealed the presence of the epitope in antigen 85 A and 85 C, suggesting cross reactivity. In ELISA and immunoblot analysis MFU50-D7 showed no 85 A binding, whereupon MFU50-D4 showed a slight cross reactivity with 85 A (85 C was not available for examination). Furthermore, a protein blast search (BLASTP, NCBI) disclosed the existence of the entire epitope in several different mycobacterial strains (i.e. *M. bovis, M. ulcerans, M. marinum, M. smegmatis, M. vaccae, etc.*), suggesting cross reactivity.

The α-85 B antibodies MFU50-A10 and MFU50-E2 recognized the epitopes “SSDPAWERNDPT” and “SSDPAWERN” respectively. This epitope region appears to possess great immunogenicity. Shen and coworkers (Shen et al. 2009) reported the synthetic peptide “GPSSDPAWERNDPTQ QIPKL” (epitope ID 21797, IEDB) was recognized by sera (IgG) of TB+ individuals. Similar peptides containing the epitope “SSDPAWERNDPT” were found to induce cytokine release or T cell proliferation in various assays with samples of TB+ individuals (Roche et al. 1994; Lightbody et al. 1998; Valle et al. 2001; D'Souza et al. 2003; Commandeur et al. 2011). The entire amino acid sequence “SSDPAWERNDPT” is present only in 85 B of *M. tuberculosis* and *M. bovis* BCG. Similar sequences are present in Mtb 85 A, 85 C, 85 D and in 85 complex proteins from other mycobacterial species (identified by BLASTP). Cross reactivity of

MFU50-A10 with Mtb 85 A, 85 D and *M. bovis* BCG cell extract and culture filtrate was detected by indirect ELISA and immunoblot. Landowski and colleagues (Landowski et al. 2001) found a “SSDPAWERNDPT” specific chicken IgY antibody to be cross reactive with Mtb 85 A and 85 C as well as 85 complex proteins of *M. avium*. Suggesting, the antibodies MFU50-A10 and MFU50-E2 could be cross reacting with Mtb 85 C and *M. avium* as well. The reaction of MFU50-A10 with 85 A, B and D indicates a possible smaller epitope than determined by epitope mapping. The amino acid sequence “DPA” is conserved throughout these 85 complex antigens, but the adjacent amino acids are differing from “SDPAWE” in 85 B to “EDPAWQ” in 85 A and “SDPAAM” in 85 D. It is possible that the epitope of MFU50-A10 is “SDPAW” or “DPAWE”, respectively two amino acids changed in 85 A or 85 D, which would explain the weaker recognition. Furthermore, the comparison of the 3D structures of antigen 85 A and 85 B (Figure 67), revealed that the glutamic acid in 85 B forms a pin structure in front of the “DPAW” region. This formation is missing in 85 A, instead of glutamic acid there is glutamine. Glutamic acid can mediate strong electrostatic attractions and hydrogen bridges through the loaded carboxylgroup, whereupon glutamine is uncharged and can only mediate hydrogen bridges through the amino- and the ketogroup (Murphy *et al.*, 2008). Thus, it is reasoned that the glutamic acid pin structure is needed structurally for correct docking, and physico-chemically for full strength binding to the antigen. In future, co-crystallization of the respective antigen with the MFU50-A10 antibody and structural analysis by X-ray diffraction could illustrate the exact epitope.

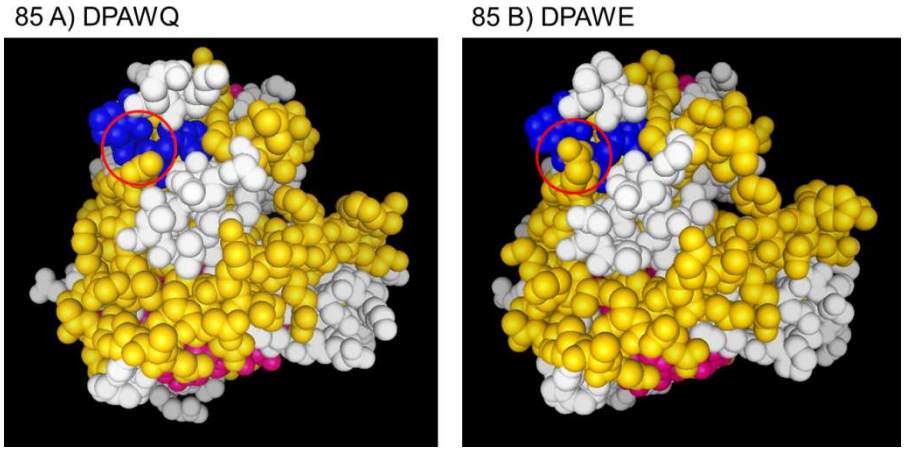


Figure 67: 3 D structures of antigens A) 85 A and B) 85 B around the amino acid sequence “DPAW”.
A) Pdb1sfr (Resolution 2.7 Å, Ronning et al. 2004) and B) Pdb1f0n (Resolution 1.9 Å, Anderson et al. 2001) were modified with 3D molecule viewer (Invitrogen). Respectively only protein chain A is shown, atoms are displayed as space filling balls, “DPAW” is marked in blue, glutamic acid in 85 B is encircled in comparison to glutamine in 85 A.

MFU50-C10 recognized the epitope “SPAVYL”, which is close to the suggested active site of antigen 85 B (Ronning *et al.*, 2000; Anderson *et al.*, 2001), offering a possible inhibitory effect by steric hindrance (Arnon 1975). Thereby, MFU50-C10 may be suitable as a TB drug. The region around the active site of 85 B seems to be of strong immunogenicity. Synthetic peptides including “SPAVYL” were found to induce cytokine release and T cell proliferation in peripheral blood mononuclear cells of TB+ humans (Valle et al. 2001; Mustafa et al. 2005; Roche et al. 1994; Commandeur et al. 2011) and antibodies against these peptides were detected in sera of TB+ individuals (Gaseitsiwe et al. 2008; Shen et al. 2009). According to BLASTP similar sequences, with substitution of one amino acid, are present in the other 85 complex proteins. Furthermore, equivalent sequences are present in several other mycobacterial species (i.e. *M. bovis*, *M. marinum*, *M. leprae*, *M. vaccae*, *M. ulcerans*, *M. avium*, etc.). No cross reactivity with 85 A and 85 D (sequence in 85 C is identical) was detected by indirect ELISA and immunoblot, suggesting specificity for antigen 85 B.

To the author’s knowledge, in this study the first human recombinant antibodies (MFU50-C10, MFU50-D7 and MFU50-E2) specific for antigen 85 B were isolated. These antibodies, in combination with the α-85 A and α-85 D antibodies generated in this work, allow discrimination between individual components of the 85 complex. Ferrara and co-workers (Ferrara et al. 2012) selected 48 antibodies directed against the Mtb 85 complex in a combination of yeast and phage display. None of these antibodies was specific for one antigen but cross reactive with all other 85 complex proteins. Landowski and colleagues (Landowski et al. 2001) isolated an α-85 B chicken IgY cross reactive with 85 complex proteins. Drowart et al. (Drowart et al. 1992) generated seven monoclonal α-85 complex antibodies, which were all cross reactive with other mycobacterial species and none was specific for antigen 85 B.

5.4.3 A-85 B sandwich ELISA

Sandwich ELISA detection of recombinant 85 B with the α-85 B scFv-Fc was successful in all performed assays. The most suitable combination, capturing with MFU50-A10 and detection with MFU50-C10-HRP, reached a detection limit of ~ 10 ng mL^{-1} (0.3 nM). Sandwich LFIA detection of recombinant 85 B was successful capturing with MFU50-A10 and detecting with MFU50-D4-gold. A detection limit of 5 ng/strip (0.03 ng/mL, 0.8 pM) was evaluated. To the author’s knowledge there are no comparable studies on antigen 85 B sandwich detection. Only whole 85 complex sandwich assays were reported. Ferrara and colleagues reported a detection limit of 6.1 nM 85 complex in PBS in a sandwich fluorescence assisted cell sorting assay with antibodies displayed on phages and yeast cells (Ferrara *et al.*, 2012). Mukundan and co-workers reported a

detection limit of 183 nM 85 complex in spiked human serum in a sandwich ELISA with monclonal antibodies (Mukundan *et al.*, 2012). Further, the presence of 85 complex in human serum was demonstrated by indirect dot immunobinding assays (Bentley-Hibbert *et al.*, 1999) and indirect ELISA (Kashyap *et al.*, 2007), but in these studies no quantification was performed.

Sandwich detection of recombinant 85 B was enhanced by multimeric antigen conformation, and reduced by monomeric antigen conformation. The small size of the antigen (~ 5 nm diameter, (Anderson *et al.*, 2001)) may be responsible for this finding. Considering the length of a human scFv of ~ 4.5 nm (Boehm *et al.*, 1999) and the Fc-mediated homodimerisation of scFv-Fc fusions (Jäger *et al.*, 2013; Powers *et al.*, 2001), sterical inhibition by the capture antibody is possible. The commercially available immunochromatographic assays for MPT64 antigen detection in Mtb cultures are sandwich assays with monoclonal antibodies (Yin *et al.*, 2013). The Capilia TB Test Kit (Ngamlert *et al.*, 2009) uses only one monoclonal antibody for a sandwich assay, indicating multimer detection. Recombinant antigen MPT64 showed multimerization by disulphide bonds in two unrelated studies (Geisbrecht *et al.*, 2006; Chu and Yuann, 2011) and no difference in immunogenicity compared to native antigen (Geisbrecht *et al.*, 2006; Oettinger *et al.*, 1997). These findings imply multimerization of native MPT64 and that these aggregates are detected in sandwich assays.

In future, antibodies suitable for native 85 B sandwich detection can be selected by modified panning on pre-bound antigen. Therefore native antigen should be captured by the antibodies presented in this work, and panning on this antigen-antibody complex, with human IgG pre-blocked phages, ought to be carried out.

5.4.4 A-85 B sandwich LFIA

All α-85 B scFv-Fc were conjugated to colloidal gold and direct detection of recombinant 85 B was possible with all conjugates. The most suitable combination for sandwich detection was evaluated to MFU50-A10 for capturing and MFU50-D4-gold for detection. Unfortunately, large production of MFU50-D4-gold conjugate led to background reactions, which were predominantly influenced by pH. Antigen detection at physiological pH 7.4 was possible (with background) and not possible at conjugation pH 8.8 (no background). These results indicate that the scFv-Fc-gold conjugate was instable at pH 7.4 and therefore loose colloidal gold particles caused background reactions. Furthermore it was reasoned that the antibodies were not able to detect antigen at pH 8.8, when the scFv-Fc-gold conjugate was stable. MFU50-A10 and MFU50-C10 scFv-Fc antibodies display theoretical isoelectric points (pI) of 8.19 and 8.64, leading to a positive

netto charge at pH 7.4. In comparison to that antigen 85 B (recombinant with C-terminal His-tag) has a pI of 6.29, resulting in a negative netto charge at physiological pH (Table 53). Probably certain charges of amino acids in the epitope areas and CDRs (Table 53) are necessary for association of the antigen-antibody complex. Further, the conformation of the antibodies at pH 8.8 probably was altered so that the reaction with the antigen was inhibited and not detectable in the explored system.

Table 53: Electrochemical properties of α-85 B scFv-Fc and antigen 85 B.

	theoretical pI*	netto charge at pH 7.4	charged aa in epitopes	charged aa in CDRs
MFU50-A10	8.19	positive	4	5
MFU50-C10	8.64	positive	0	13
MFU50-D4	8.18	positive	1	5
MFU50-D7	7.11	negative	2	11
MFU50-E2	8.50	positive	3	4
85 B (C-His)	6.29	negative		

*determined with the ExPASY Prot Param tool (Artimo *et al.*, 2012)

Background reactions were minimized with preserved antigen recognition by addition of a diluent. With this optimized assay a detection limit of 5 ng recombinant 85 B per strip in 7H9 medium was reached. No reaction with Mtb cell extract or culture filtrates (concentrated and un-concentrated) was observed. Similarly to the sandwich ELISA, lack of multimers in the explored samples may be responsible for these findings. In addition, even if multimers are present, the 85 B proteins can be arranged in an epitope blocking conformation.

Furthermore, human urine and sputum samples were spiked with recombinant 85 B and analysed by sandwich LFIA. The spike was fully retrieved from the urine samples, but only to 50 % from sputum samples. Proteases present in sputum may be responsible for protein degradation and therefore lack of spike recovery. The presence of the 85 complex was demonstrated in human serum (Kashyap et al. 2007), urine (Bentley-Hibbert et al. 1999), cerebrospinal fluid (Kashyap et al. 2005) and sputum (Wallis et al. 1998). All available sputum/urine samples of confirmed TB patients were autoclaved prior to storage at Lionex GmbH. Previous experiments with boiled 85 B showed decreased antigen recognition in ELISA compared to untreated 85 B. If there has been a detectable amount of 85 B in the sputum samples, it was probably destroyed by autoclaving. In addition to that, considering the presence of proteases in sputum and the storage of the samples at 2 – 8 °C over years, it was assumed that 85 B not affected by autoclaving would have been already digested.

5.4.5 A-85 B immunoblot

Detection of 85 B in Mtb culture filtrates was difficult probably due to antigen conformation and/or amount of antigen in previously described assays. The antibodies used in this assays were selected by panning on recombinant 85 B produced in *E. coli*. Although 85 complex proteins do not require posttranslational modifications, differences in protein folding may occur. Further, 85 B contains an N-terminal signal sequence (Borremans *et al.*, 1989), which is not processed in *E. coli* but at least partially cleaved for secretion in *M. tuberculosis*. All α-85 B scFv-Fc recognized continuous epitopes. Therefore antigen detection in samples, mostly independent from conformation, by an immunoblot assay was considered possible. Indeed, after 22 days of cultivation 85 B was detected in 20 fold Mtb culture filtrate by MFU50-A10-HRP. No reaction with MFU50-C10-HRP and MFU50-D4-HRP was detected. As mentioned before, MFU50-A10 is cross reactive with antigens 85 A and 85 D (probably 85 C), which can lead to an advantage over MFU50-C10 and MFU50-D4 that are specific for 85 B. Commercial MPT64 antigen detection is mostly performed from un-concentrated liquid cultures of *M. tuberculosis* grown in minimal medium for approximately 2 – 3 weeks (Abe *et al.*, 1999). Thereby, MPT64 detection is as fast as the presented 85 B detection, but more sensitive. The α-MPT64 monoclonal IgG antibodies in commercial kits are affinity matured, in comparison to the naïve recombinant antibodies used here. The affinities of the α-85 B antibodies could not be evaluated by surface plasmon resonance due to the multimeric conformation of the recombinant antigen (data not shown). Affinities of scFv derived from the HAL7/8 libraries are usually in the range of 10^{-7} to 10^{-10} M^{-1} (personal communication with M. Hust). *In vitro* affinity maturation by construction of secondary libraries, introducing additional mutations, and following selection by increased selective pressure in phage display could improve the affinities of the explored antibodies to picomolar range (Thie *et al.*, 2009; Steidl *et al.*, 2008). Furthermore, the presented immunoblot assay may be suitable for detection of 85 B in human serum. Landowski and colleagues (Landowski et al. 2001) generated an oligoclonal chicken IgY antibody against 85 complex and demonstrated 55 % sensitivity and 85 % specificity for detection of circulating 85 complex in human blood by an immunoblot approach.

5.5 85 A

Four individual α-85 A scFv were solated from HAL7/8 and purified as scFv-Fc antibodies. All antibodies were strongly reactive with reduced 85 A in immunoblot, therefore recognition of a continuous epitope was supposed. Epitope mapping was carried out by screening overlapping 85 A peptides immobilized on a cellulose membrane. The antibody MFU53-F3 was reacting with a great variety of peptides. Such observations usually indicate a discontinuous epitope. Furthermore, this antibody showed strong cross reactivity in immunoblot and ELISA with 85 B, but no reaction with 85 D. Suggesting, a conformational epitope is recognized, which is present in 85 A and 85 B but absent in 85 D. In addition, unspecific reactions with BSA at concentrations above 200 ng/mL were encountered in ELISA. However, unspecificity can be provoked by high antibody concentrations. Therefore, it remains unclear which kind of epitope MFU53-F3 recognizes and how specific the binding is. For the other α-85 A antibodies the epitopes were determined. MFU12-D8 recognized the sequence “SPA” and MFU53-A3 recognized the adjacent sequence “LYLLDG”. Interestingly, the α-85 B antibody MFU50-C10 recognized a similar sequence “SPAVYL”. Like MFU50-C10 for 85 B, the α-85 A antibody MFU12-D8 showed no cross reactions with the other 85 complex proteins, neither in ELISA nor in immunoblot, and seems to be specific for 85 A. The amino acids “SPA” in both antigens form identical structures and are located in a cavity, yet they are surrounded by a ring of different amino acids (Figure 68). No reactions with those surrounding amino acids were detected in epitope mapping. The linear epitope “SPA” seems to be complemented by the structurally surrounding amino acid residues.

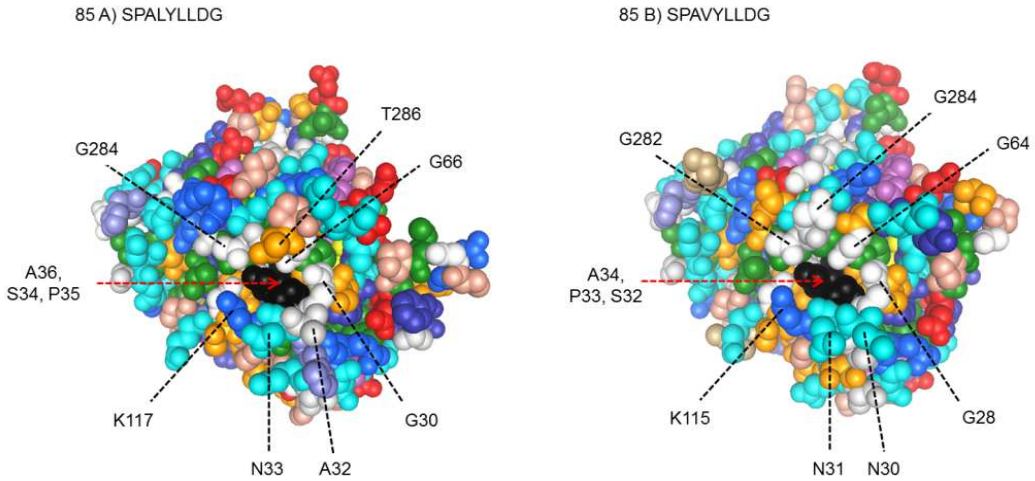


Figure 68: 3 D structures of antigens A) 85 A and B) 85 B around the amino acid sequence “SPA”.
A) Pdb1sfr protein chain A (Resolution 2.7 Å, Ronning et al. 2004) and B) Pdb1f0n protein chain B (Resolution 1.9 Å, Anderson et al. 2001) were modified with 3D molecule viewer (Invitrogen). Atoms are displayed as space filling balls, different amino acids are displayed with different colours, “SPAnYLLDG” is marked in black.

Further, the sequence “SPALYLLDG” (aa 76 – 84) is interpenetrating a part of antigen 85 A, whereat “SPA” is located at one site and “LYLLDG” at the other site. The “SPA” (aa 76 - 78) cavity is close to the catalytic serin 126 (Ronning *et al.*, 2004), which may be sterically blocked by binding of MFU12-D8. Furthermore, the “LYLLDG” cavity is located in the carbohydrate binding pocket of antigen 85 A and could also mediate sterical inhibition. Thereby, considering the importance of 85 A in Mtb growth (Armitige *et al.*, 2000; Belisle *et al.*, 1997), MFU12-D8 and MFU53-A3 may be suitable as TB drugs.

Sandwich ELISA detection of recombinant 85 A was possible with all antibody combinations. Similar to antigen 85 B, multimeric antigen conformation seemed to enhance sandwich detection. For antigen 85 A; only two epitope areas were determined with close proximity (2 - 3 nm). Once monomeric antigen was bound by the capture antibody, the other epitope could be directed towards the surface of the MTP, and hence sterically prohibit sandwich detection (Figure 69).

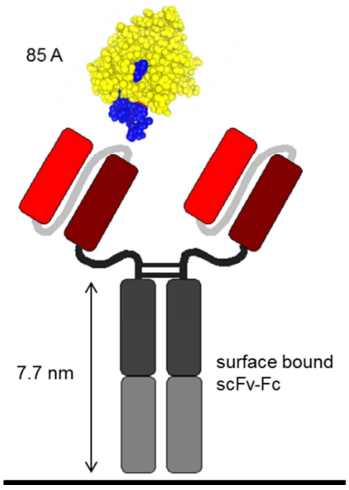


Figure 69: Possible sterical inhibition of 85 A sandwich binding.
Pdb1sfr (Resolution 2.7 Å, Ronning et al. 2004) was modified with 3D molecule viewer (Invitrogen). Only protein chain A is shown (yellow), atoms are displayed as space filling balls, epitopes are marked in blue. Antibody symbol adopted from M. Hust with permission.

In future, antibodies suitable for sandwich detection of native 85 A can be isolated by panning on pre-bound antigen, and the biochemical charcteristics of these antibodies can be altered, as described for the α-85 B antibodies.

In this work, antibodies specifically binding to Mtb antigens 85 A, 85 B or 85 D were isolated. To the author’s knowledge, there are only studies presenting antibodies cross reacting with minimum one other antigen of the 85 complex (Ferrara *et al.*, 2012; Landowski *et al.*, 2001; Drowart *et al.*, 1992). Thereby, with the presented antibodies, discrimination between individual components of the 85 complex is possible for the first

time. Easy applicability of the presented antibodies to *in vitro* experiments was showed in this study by conjugation to colloidal gold or HRP. Due to the recombinant nature of the explored antibodies, they can easily be altered to different formats, fused to different Fc-parts or fused to markers such as green fluorescent protein (GFP, (Kremer *et al.*, 1995)) for *in vivo* experiments. Thus the role of particular 85 proteins in Mtb cell wall biosynthesis and evasion of the host's immune response may be explored.

6 References

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8 Appendix

8.1 Plasmid maps

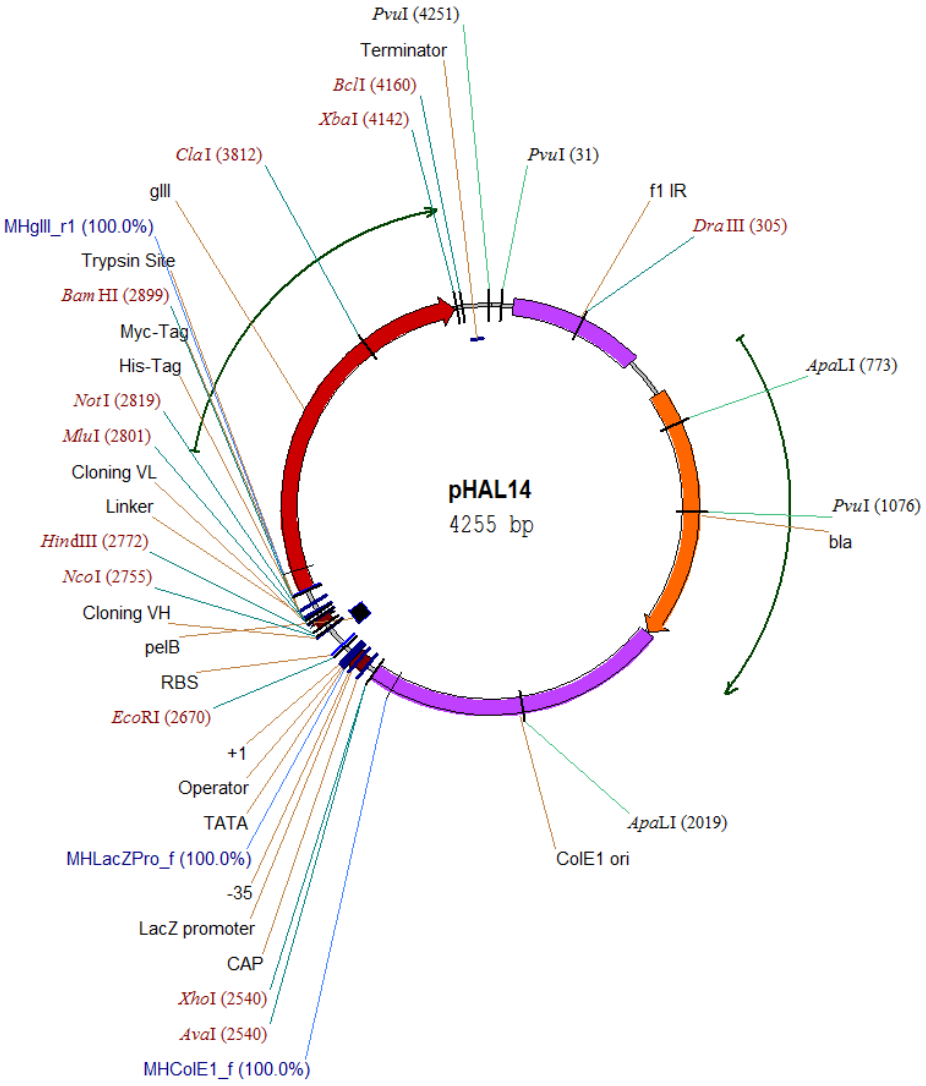

Figure 70: Plasmid map for pHAL14.
Vector for phage display of scFv (Hust *et al.*, 2011).

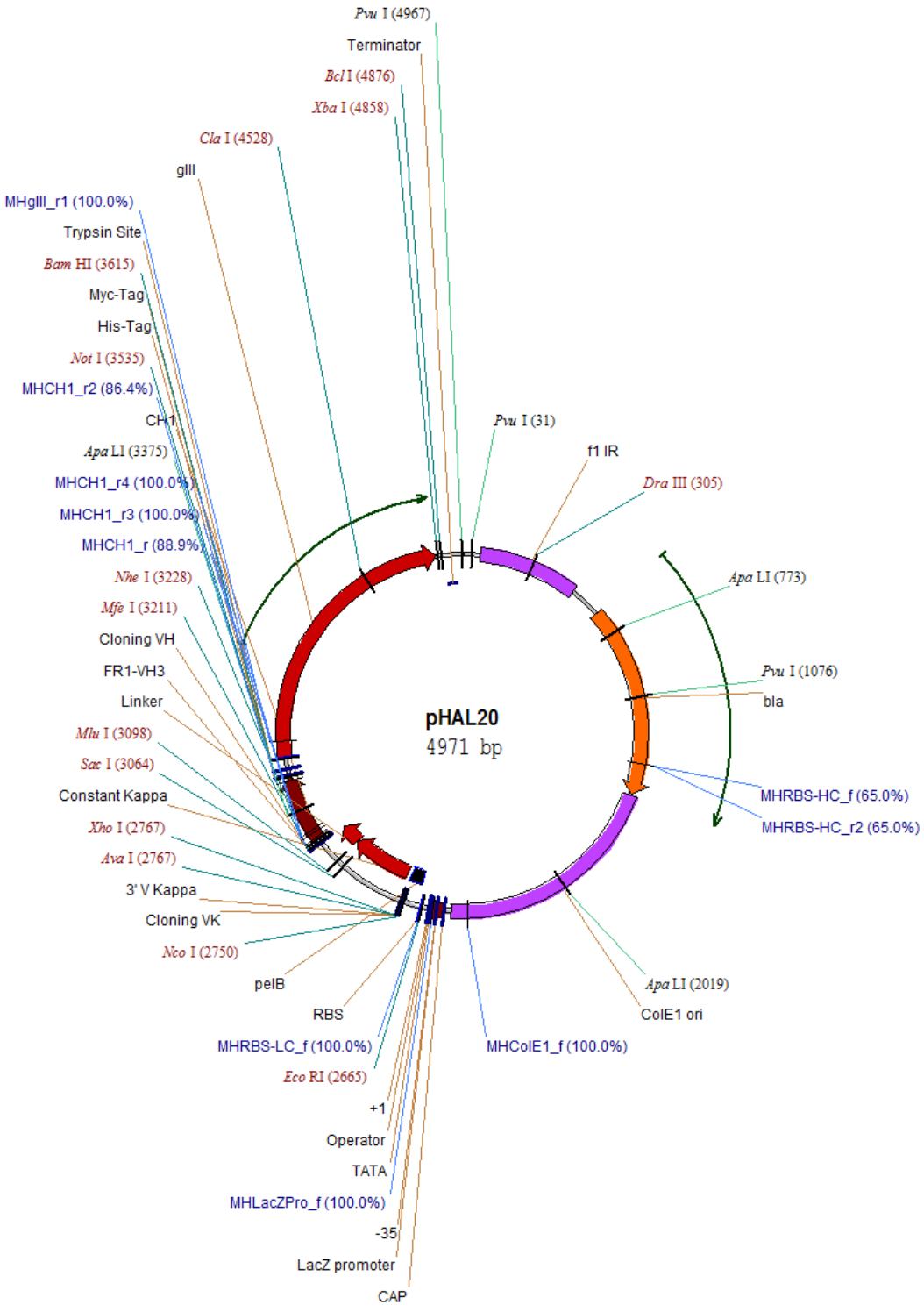

Figure 71: Plasmid map for pHAL20.
Vector for phage display of scFab (M. Hust unpublished)

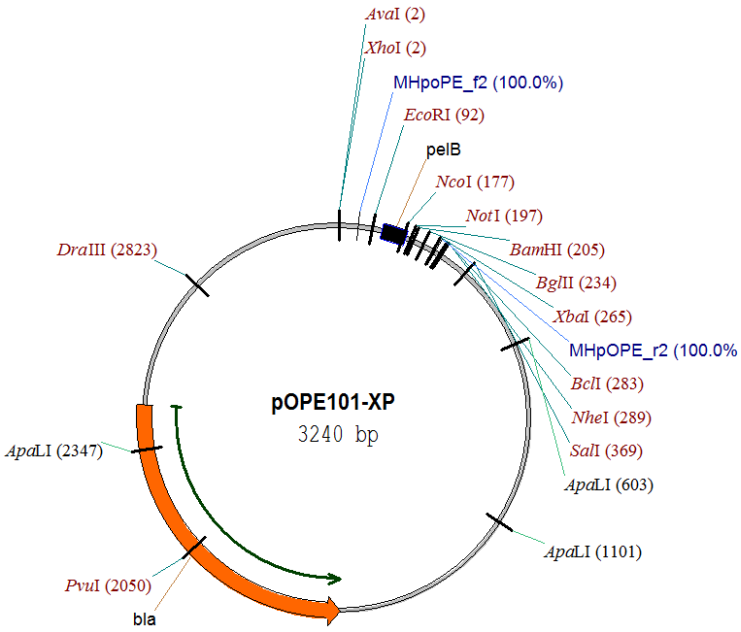

Figure 72: Plasmid map for pOPE101-XP.
Vector for scFv and scFab expression in prokaryotic cells (*E. coli*) (Hust *et al.*, 2009).

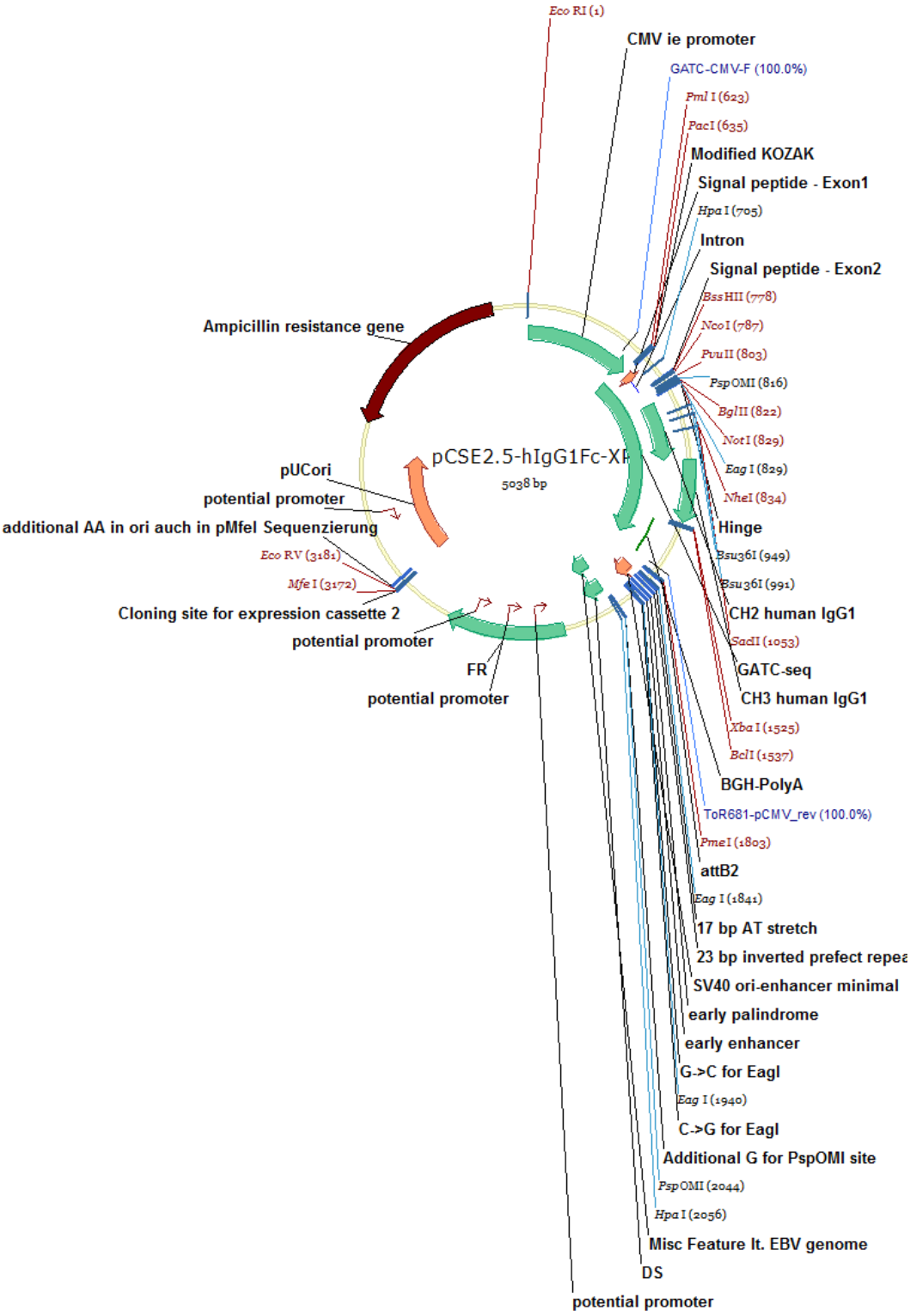

Figure 73: Plasmid map for pCSE2.5-hIgG1-Fc-XP.
Vector for scFv-Fc and scFab-Fc expression in eukaryotic cells (HEK293-6E) (Jäger *et al.*, 2013).

8.2 Peptides for epitope mapping

Table 54: Peptide sequences on 16 kDa peptide-spot membrane.

spot	start aa	peptide sequence	spot	start aa	peptide sequence
1	1	MATTLPVQRHPRSLF	**19**	73	GQLTIKAERTEQKDF
2	5	LPVQRHPRSLFPEFS	**20**	77	IKAERTEQKDFDGRS
3	9	RHPRSLFPEFSELFA	**21**	81	RTEQKDFDGRSEFAY
4	13	SLFPEFSELFAAFPS	**22**	85	KDFDGRSEFAYGSFV
5	17	EFSELFAAFPSFAGL	**23**	89	GRSEFAYGSFVRTVS
6	21	LFAAFPSFAGLRPTF	**24**	93	FAYGSFVRTVSLPVG
7	25	FPSFAGLRPTFDTRL	**25**	97	SFVRTVSLPVGADED
8	29	AGLRPTFDTRLMRLE	**26**	101	TVSLPVGADEDDIKA
9	33	PTFDTRLMRLEDEMK	**27**	105	PVGADEDDIKATYDK
10	37	TRLMRLEDEMKEGRY	**28**	109	DEDDIKATYDKGILT
11	41	RLEDEMKEGRYEVRA	**29**	113	IKATYDKGILTVSVA
12	45	EMKEGRYEVRAELPG	**30**	117	YDKGILTVSVAVSEG
13	49	GRYEVRAELPGVDPD	**31**	121	ILTVSVAVSEGKPTE
14	53	VRAELPGVDPDKDVD	**32**	125	SVAVSEGKPTEKHIQ
15	57	LPGVDPDKDVDIMVR	**33**	129	SEGKPTEKHIQIRST
16	61	DPDKDVDIMVRDGQL	**34**	133	PTEKHIQIRSTN
17	65	DVDIMVRDGQLTIKA	**35**	137	HIQIRSTN
18	69	MVRDGQLTIKAERTE	**36**	141	RSTN

Table 55: Peptide sequences on CFP-10 peptide-spot membrane.

spot	start aa	peptide sequence	spot	start aa	peptide sequence
1	1	MAEMKTDAATLAQEA	**16**	46	AAGTAAQAAVVRFQE
2	4	MKTDAATLAQEAGNF	**17**	49	TAAQAAVVRFQEAAN
3	7	DAATLAQEAGNFERI	**18**	52	QAAVVRFQEAANKQK
4	10	TLAQEAGNFERISGD	**19**	55	VVRFQEAANKQKQEL
5	13	QEAGNFERISGDLKT	**20**	58	FQEAANKQKQELDEI
6	16	GNFERISGDLKTQID	**21**	61	AANKQKQELDEISTN
7	19	ERISGDLKTQIDQVE	**22**	64	KQKQELDEISTNIRQ
8	22	SGDLKTQIDQVESTA	**23**	67	QELDEISTNIRQAGV
9	25	LKTQIDQVESTAGSL	**24**	70	DEISTNIRQAGVQYS
10	28	QIDQVESTAGSLQGQ	**25**	73	STNIRQAGVQYSRAD
11	31	QVESTAGSLQGQWRG	**26**	76	IRQAGVQYSRADEEQ
12	34	STAGSLQGQWRGAAG	**27**	79	AGVQYSRADEEQQQA
13	37	GSLQGQWRGAAGTAA	**28**	82	QYSRADEEQQQALSS
14	40	QGQWRGAAGTAAQAA	**29**	85	RADEEQQQALSSQMG
15	43	WRGAAGTAAQAAVVR	**30**	88	ADEEQQQALSSQMGF

Table 56: Peptide sequences on 85 A peptide-spot membrane.

spot	start aa	peptide sequence	spot	start aa	peptide sequence
1	1	MQLVDRVRGAVTGMS	**56**	166	VGLSMAASSALTLAI
2	4	VDRVRGAVTGMSRRL	**57**	169	SMAASSALTLAIYHP
3	7	VRGAVTGMSRRLVVG	**58**	172	ASSALTLAIYHPQQF
4	10	AVTGMSRRLVVGAVG	**59**	175	ALTLAIYHPQQFVYA
5	13	GMSRRLVVGAVGAAL	**60**	178	LAIYHPQQFVYAGAM
6	16	RRLVVGAVGAALVSG	**61**	181	YHPQQFVYAGAMSGL
7	19	VVGAVGAALVSGLVG	**62**	184	QQFVYAGAMSGLLDP
8	22	AVGAALVSGLVGAVG	**63**	187	VYAGAMSGLLDPSQA
9	25	AALVSGLVGAVGGTA	**64**	190	GAMSGLLDPSQAMGP
10	28	VSGLVGAVGGTATAG	**65**	193	SGLLDPSQAMGPTLI
11	31	LVGAVGGTATAGAFS	**66**	196	LDPSQAMGPTLIGLA
12	34	AVGGTATAGAFSRPG	**67**	199	SQAMGPTLIGLAMGD
13	37	GTATAGAFSRPGLPV	**68**	202	MGPTLIGLAMGDAGG
14	40	TAGAFSRPGLPVEYL	**69**	205	TLIGLAMGDAGGYKA
15	43	AFSRPGLPVEYLQVP	**70**	208	GLAMGDAGGYKASDM
16	46	RPGLPVEYLQVPSPS	**71**	211	MGDAGGYKASDMWGP
17	49	LPVEYLQVPSPSMGR	**72**	214	AGGYKASDMWGPKED
18	52	EYLQVPSPSMGRDIK	**73**	217	YKASDMWGPKEDPAW
19	55	QVPSPSMGRDIKVQF	**74**	220	SDMWGPKEDPAWQRN
20	58	SPSMGRDIKVQFQSG	**75**	223	WGPKEDPAWQRNDPL
21	61	MGRDIKVQFQSGGAN	**76**	226	KEDPAWQRNDPLLNV
22	64	DIKVQFQSGGANSPA	**77**	229	PAWQRNDPLLNVGKL
23	67	VQFQSGGANSPALYL	**78**	232	QRNDPLLNVGKLIAN
24	70	QSGGANSPALYLLDG	**79**	235	DPLLNVGKLIANNTR
25	73	GANSPALYLLDGLRA	**80**	238	LNVGKLIANNTRVWV
26	76	SPALYLLDGLRAQDD	**81**	241	GKLIANNTRVWVYCG
27	79	LYLLDGLRAQDDFSG	**82**	244	IANNTRVWVYCGNGK
28	82	LDGLRAQDDFSGWDI	**83**	247	NTRVWVYCGNGKPSD
29	85	LRAQDDFSGWDINTP	**84**	250	VWVYCGNGKPSDLGG
30	88	QDDFSGWDINTPAFE	**85**	253	YCGNGKPSDLGGNNL
31	91	FSGWDINTPAFEWYD	**86**	256	NGKPSDLGGNNLPAK
32	94	WDINTPAFEWYDQSG	**87**	259	PSDLGGNNLPAKFLE
33	97	NTPAFEWYDQSGLSV	**88**	262	LGGNNLPAKFLEGFV
34	100	AFEWYDQSGLSVVMP	**89**	265	NNLPAKFLEGFVRTS
35	103	WYDQSGLSVVMPVGG	**90**	268	PAKFLEGFVRTSNIK
36	106	QSGLSVVMPVGGQSS	**91**	271	FLEGFVRTSNIKFQD
37	109	LSVVMPVGGQSSFYS	**92**	274	GFVRTSNIKFQDAYN
38	112	VMPVGGQSSFYSDWY	**93**	277	RTSNIKFQDAYNAGG
39	115	VGGQSSFYSDWYQPA	**94**	280	NIKFQDAYNAGGGHN
40	118	QSSFYSDWYQPACGK	**95**	283	FQDAYNAGGGHNGVF
41	121	FYSDWYQPACGKAGC	**96**	286	AYNAGGGHNGVFDFP
42	124	DWYQPACGKAGCQTY	**97**	289	AGGGHNGVFDFPDSG
43	127	QPACGKAGCQTYKWE	**98**	292	GHNGVFDFPDSGTHS
44	130	CGKAGCQTYKWETFL	**99**	295	GVFDFPDSGTHSWEY
45	133	AGCQTYKWETFLTSE	**100**	298	DFPDSGTHSWEYWGA
46	136	QTYKWETFLTSELPG	**101**	301	DSGTHSWEYWGAQLN
47	139	KWETFLTSELPGWLQ	**102**	304	THSWEYWGAQLNAMK
48	142	TFLTSELPGWLQANR	**103**	307	WEYWGAQLNAMKPDL
49	145	TSELPGWLQANRHVK	**104**	310	WGAQLNAMKPDLQRA
50	148	LPGWLQANRHVKPTG	**105**	313	QLNAMKPDLQRALGA
51	151	WLQANRHVKPTGSAV	**106**	316	AMKPDLQRALGATPN
52	154	ANRHVKPTGSAVVGL	**107**	319	PDLQRALGATPNTGP
53	157	HVKPTGSAVVGLSMA	**108**	322	QRALGATPNTGPAPQ
54	160	PTGSAVVGLSMAASS	**109**	324	ALGATPNTGPAPQGA
55	163	SAVVGLSMAASSALT			

Table 57: Peptide sequences on 85 B peptide-spot membrane.

spot	start aa	peptide sequence	spot	start aa	peptide sequence
1	1	MTDVSRKIRAWGRRL	**54**	160	SAAIGLSMAGSSAMI
2	4	VSRKIRAWGRRLMIG	**55**	163	IGLSMAGSSAMILAA
3	7	KIRAWGRRLMIGTAA	**56**	166	SMAGSSAMILAAYHP
4	10	AWGRRLMIGTAAAVV	**57**	169	GSSAMILAAYHPQQF
5	13	RRLMIGTAAAVVLPG	**58**	172	AMILAAYHPQQFIYA
6	16	MIGTAAAVVLPGLVG	**59**	175	LAAYHPQQFIYAGSL
7	19	TAAAVVLPGLVGLAG	**60**	178	YHPQQFIYAGSLSAL
8	22	AVVLPGLVGLAGGAA	**61**	181	QQFIYAGSLSALLDP
9	25	LPGLVGLAGGAATAG	**62**	184	IYAGSLSALLDPSQG
10	28	LVGLAGGAATAGAFS	**63**	187	GSLSALLDPSQGMGP
11	31	LAGGAATAGAFSRPG	**64**	190	SALLDPSQGMGPSLI
12	34	GAATAGAFSRPGLPV	**65**	193	LDPSQGMGPSLIGLA
13	37	TAGAFSRPGLPVEYL	**66**	196	SQGMGPSLIGLAMGD
14	40	AFSRPGLPVEYLQVP	**67**	199	MGPSLIGLAMGDAGG
15	43	RPGLPVEYLQVPSPS	**68**	202	SLIGLAMGDAGGYKA
16	46	LPVEYLQVPSPSMGR	**69**	205	GLAMGDAGGYKAADM
17	49	EYLQVPSPSMGRDIK	**70**	208	MGDAGGYKAADMWGP
18	52	QVPSPSMGRDIKVQF	**71**	211	AGGYKAADMWGPSSD
19	55	SPSMGRDIKVQFQSG	**72**	214	YKAADMWGPSSDPAW
20	58	MGRDIKVQFQSGGNN	**73**	217	ADMWGPSSDPAWERN
21	61	DIKVQFQSGGNNSPA	**74**	220	WGPSSDPAWERNDPT
22	64	VQFQSGGNNSPAVYL	**75**	223	SSDPAWERNDPTQQI
23	67	QSGGNNSPAVYLLDG	**76**	226	PAWERNDPTQQIPKL
24	70	GNNSPAVYLLDGLRA	**77**	229	ERNDPTQQIPKLVAN
25	73	SPAVYLLDGLRAQDD	**78**	232	DPTQQIPKLVANNTR
26	76	VYLLDGLRAQDDYNG	**79**	235	QQIPKLVANNTRLWV
27	79	LDGLRAQDDYNGWDI	**80**	238	PKLVANNTRLWVYCG
28	82	LRAQDDYNGWDINTP	**81**	241	VANNTRLWVYCGNGT
29	85	QDDYNGWDINTPAFE	**82**	244	NTRLWVYCGNGTPNE
30	88	YNGWDINTPAFEWYY	**83**	247	LWVYCGNGTPNELGG
31	91	WDINTPAFEWYYQSG	**84**	250	YCGNGTPNELGGANI
32	94	NTPAFEWYYQSGLSI	**85**	253	NGTPNELGGANIPAE
33	97	AFEWYYQSGLSIVMP	**86**	256	PNELGGANIPAEFLE
34	100	WYYQSGLSIVMPVGG	**87**	259	LGGANIPAEFLENFV
35	103	QSGLSIVMPVGGQSS	**88**	262	ANIPAEFLENFVRSS
36	106	LSIVMPVGGQSSFYS	**89**	265	PAEFLENFVRSSNLK
37	109	VMPVGGQSSFYSDWY	**90**	268	FLENFVRSSNLKFQD
38	112	VGGQSSFYSDWYSPA	**91**	271	NFVRSSNLKFQDAYN
39	115	QSSFYSDWYSPACGK	**92**	274	RSSNLKFQDAYNAAG
40	118	FYSDWYSPACGKAGC	**93**	277	NLKFQDAYNAAGGHN
41	121	DWYSPACGKAGCQTY	**94**	280	FQDAYNAAGGHNAVF
42	124	SPACGKAGCQTYKWE	**95**	283	AYNAAGGHNAVFNFP
43	127	CGKAGCQTYKWETFL	**96**	286	AAGGHNAVFNFPPNG
44	130	AGCQTYKWETFLTSE	**97**	289	GHNAVFNFPPNGTHS
45	133	QTYKWETFLTSELPQ	**98**	292	AVFNFPPNGTHSWEY
46	136	KWETFLTSELPQWLS	**99**	295	NFPPNGTHSWEYWGA
47	139	TFLTSELPQWLSANR	**100**	298	PNGTHSWEYWGAQLN
48	142	TSELPQWLSANRAVK	**101**	301	THSWEYWGAQLNAMK
49	145	LPQWLSANRAVKPTG	**102**	304	WEYWGAQLNAMKGDL
50	148	WLSANRAVKPTGSAA	**103**	307	WGAQLNAMKGDLQSS
51	151	ANRAVKPTGSAAIGL	**104**	310	QLNAMKGDLQSSLGA
52	154	AVKPTGSAAIGLSMA	**105**	311	LNAMKGDLQSSLGAG
53	157	PTGSAAIGLSMAGSS			